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Irreversible inhibition of rat glutathione *S*-transferase 1–1 by quinones and their glutathione conjugates

Structure–activity relationship and mechanism

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The irreversible inhibition of the rat glutathione *S*-transferase (GST) isoenzyme 1–1 by a series of halogenated 1,4-benzoquinones and their GSH conjugates was studied quantitatively by analysing the time course of enzyme inactivation. With increasing numbers of chlorine substituents, the rate of inhibition greatly increased. Incorporation of a GSH moiety in all cases increased the rate of inactivation compared with the non-substituted compound, and this was due to the increased affinity of the inhibitor for the active site. The ratio between the rates of inhibition for a given quinone with and without GSH substituent was largest for the three dichlorobenzoquinones, with the 2,6-isomer showing a 41-fold increase in rate of inhibition upon conjugation with GSH. The time courses of inhibition could be fitted either to a bi-exponential function (for the GSH conjugates and the higher chlorinated quinones) or to a mono-exponential function (all other quinones). It is concluded that the second component describes the affinity part of the reaction. GST 1–1 possesses two cysteine residues, with modification of one of these, probably located in the vicinity of the active site, having a major impact on the enzyme activity. Compounds with affinity towards the active site preferentially react with this residue. Non-specific quinones react equally with both cysteine residues. This was confirmed by the observation that complete inactivation of GST 1–1 by 2,5-dichlorobenzoquinone was achieved only after modification of two residues, whereas the corresponding GSH conjugate already completely inhibited the enzyme after modification of one residue.

INTRODUCTION

The glutathione *S*-transferases (GSTs) are a group of isoenzymes involved in the detoxification of alkylating electrophilic compounds, usually by catalysing the conjugation of these compounds with GSH [1]. Apart from their role in handling xenobiotics, a number of endogenous functions have been described, among which are some key steps in prostaglandin and leukotriene biosynthesis [2,3]. Furthermore, an involvement in multi-drug-resistance against cytostatics has been suggested as a result of observations that (i) GSTs are often over-expressed in tumours and (ii) alkylating cytostatics are potential substrates for GSTs [4]. GSTs are most abundantly present in liver, but are also detected in almost all other organs. They are inducible by a large variety of substances. The isoenzymes can be grouped in three classes, Alpha, Mu and Pi, according to both physiological and structural similarities. This division is valid for human, rat and mouse GSTs [5,6].

A large research effort has been made on the subject of diversity and distribution of GSTs: a large amount of information is available on the tissue distribution, substrate selectivities, inducibility and primary structure. Relatively little is known, however, about the catalytic mechanism and topology of the active site. The active site is composed of a binding site for GSH and one for the second, electrophilic, substrate [5]. The critical steps in the catalysis of the conjugation reaction are largely in the field of speculation, although the involvement of a histidine residue has been indicated [7,8].

It is known that modification of thiol groups of GST results in (partial) inactivation of the enzyme. GST 1–1 can be inactivated to various extents by several thiol-blocking reagents [9].

Recently, a specific covalently binding class of inhibitors has been developed for GST, namely the GSH conjugates of halogenated quinones. These compounds combine a reactivity towards thiol groups with affinity for the GSH-binding site, and appear to be targeted to the GST active site and subsequently react with a cysteine residue [10]. The inactivation is not due to interference with the catalytic mechanism, i.e. a reaction with amino acid residues involved in the catalysis, but is thought to result from steric hindrance of the active site [8]. Of these compounds, the GSH conjugate of tetrachloro-1,4-benzoquinone has been most extensively studied [8]. Furthermore, a number of chlorinated benzoquinones and naphthoquinones has been assayed for their inhibitory action towards rat GST [11]. The present study was designed to evaluate quantitatively the structure–inhibition relationships of halogenated quinones and the corresponding GSH conjugates. The degree of halogenation, the targeting effect of the GSH moiety and the position of the substituents are taken into account.

METHODS

Materials

BQ was from Janssen Pharmaceuticals, Beerse, Belgium. BrBQ, ClHQ, 2,5-diClBQ and 2,6-diClBQ were obtained from Eastman–Kodak, Rochester, NY, U.S.A. 2,3-diClBQ was kindly supplied by Professor U. Kückländer, Institüt für Pharmazeutische Chemie, University of Düsseldorf, Germany. TriClBQ was generously given by Mr. B. Spenkelink, Department of Toxicology, Agricultural University, Wageningen, The Netherlands. TetraClBQ was from Merck, Darmstadt, Germany.

All abbreviations used for the compounds are a composite of the following segments: BQ, 1,4-benzoquinone; HQ, 1,4-hydroquinone; GS, glutathionyl; Cl, chloro; Br, bromo. Other abbreviation used: GST, glutathione *S*-transferase.

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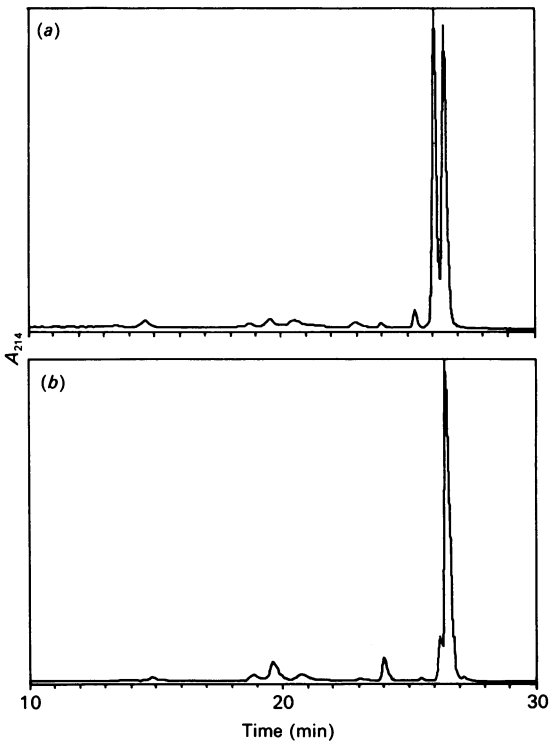

Fig. 1. H.p.l.c. analysis of the two purified forms of GST

(*a*) The heterologous form, as used for all experiments; (*b*) the form containing only the latter of the two peaks.

Table 1. Physical characteristics of GSH conjugates of halogenated quinones

All absorption maxima between 220 nm and 370 nm are presented; absorption maxima of shoulders are given in parentheses. M_r values were determined by fast-atom-bombardment m.s. This technique reduces and protonates the benzoquinones, resulting in an $M+3$ mass for GSH conjugates of benzoquinones. The redox potentials ($E_{\frac{1}{2}}$) were determined by cyclic voltametry. Abbreviation: N.M., not measured.

Quinone	$\lambda_{max.}$ (nm)	M_r	$E_{\frac{1}{2}}$
GSBQ	254	416.0	115
GSHQ	250		
2-Cl-6-GSBQ	300	450.0	100
2-Cl-6-GSHQ	N.M.		
2,3-diCl-5-GSBQ	256, 296	483.9	105, −300
2,3-diCl-5-GSHQ	317		
2,5-diCl-3-GSBQ	247, 275	483.9	120, −360
2,5-diCl-3-GSHQ	313		
2,6-diCl-3-GSBQ	247, 278	484.0	85, −450
2,6-diCl-3-GSHQ	311		
triClGSBQ	249, 288, 327	518.0	165
triClGSHQ	317		
2-Br-3-GSBQ	252		105, −85
2-Br-3-GSHQ	(262), 308	*	
2-Br-5-GSBQ	312		150, −60
2-Br-5-GSHQ	(252), 307	*	
2-Br-6-GSBQ	310		105, −25
2-Br-6-GSHQ	(250), 305	*	

* Published previously [13].

Pentachloro[^{14}C]phenol (sp. radioactivity 37 Ci/mol) was obtained from C.E.A., Gif-sur-Ivette, France. Tetrachloro-1,4-[^{14}C]benzoquinone (tetraCl[^{14}C]BQ) was prepared from pentachloro[^{14}C]phenol by cytochrome *P*-450-mediated hydroxylation and subsequent oxidation by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone, as described previously [12].

Synthesis of GSH conjugates

GSBQ was prepared by mixing equimolar amounts (1 mmol) of GSH (in 50 ml of water) and BQ (in 50 ml of methanol) and incubation for 200 min at room temperature, after which the methanol was evaporated. DDQ (50 μg) was added to oxidize the GSHQ formed, and two extractions with ethyl acetate were performed. The extracts were discarded, and the aqueous layer was concentrated to a volume of 0.6 ml. GSBQ was purified by preparative h.p.l.c. [Zorbax ODS (9.4 mm × 250 mm), flow rate 3 ml/min, with a gradient of 10–60 % (v/v) methanol in aq. 1 % (v/v) formic acid in 10 min (k' of GSBQ was approx. 3)].

All diClGSBQ isomers were synthesized by adding, with vigorous shaking, 0.2 mmol of GSH in 2 ml of water to 2 mmol of the diClBQ compounds in 200 ml of methanol. After 10 min of incubation, the solvents were evaporated, 100 ml of water was added and the unchanged quinones and hydroquinones were extracted with ethyl acetate (three times). For the 2,3- and the 2,5-isomer the sole product was the GSdiClBQ. In the case of the 2,6-isomer, a mixture of 2,6-diClGSBQ and 2-Cl-6-GSBQ was formed. These compounds were separated by preparative h.p.l.c. [Zorbax ODS (9.6 mm × 250 mm), flow rate 2 ml/min, with a gradient of 30–80 % (v/v) methanol in aq. 1 % (v/v) formic acid, pH 2.5, in 25 min (k' of 2,6-diClGSBQ was 16.5 and k' of 2-Cl-6-GSBQ was 12.6)].

The synthesis of GStetraClBQ has been published previously [10]. The synthesis of GStriClBQ was identical with that for its tetrachloro analogue. The synthesis of the three GSBrHQ isomers has been described elsewhere [13]. The oxidation of the hydroquinone to the benzoquinone form of the bromo compounds was achieved by adding a 2-fold molar excess of Ag_2O to a 1 mg/ml solution of the hydroquinones in water and incubating for 30 min at 0 °C, followed by centrifugation at 1000 *g* for 5 min to remove solid Ag_2O. H.p.l.c. confirmed the purity of the product [Lichrosorb ODS (2.5 mm × 100 mm), flow rate 0.6 ml/min, with a gradient of 20–60 % (v/v) methanol in 10 min, with aq. 1 % (v/v) acetic acid as counter-eluent (k' values of all GSH conjugates of bromobenzoquinone were approx. 8)].

All compounds were analysed for purity and identity by fast-atom-bombardment m.s., absorption spectroscopy and h.p.l.c., as summarized in the Results section. The characteristics of the quinones and their respective GSH conjugates are presented in Table 1. The oxidized nature of all quinones was confirmed by h.p.l.c. analysis with and without added reducing agent (ascorbic acid). All inhibitors were over 95 % pure, as judged by h.p.l.c.–diode-array analysis (220–370 nm), with only the corresponding hydroquinone or isomeric forms detected as impurities.

Purification of GST 1-1

GST 1-1 was purified from hepatic cytosol from phenobarbital-induced male Sprague–Dawley rats [15 weeks of age; treated for 7 days with 0.1 % (w/v) solution in drinking water]. A mixture of the purified major GSTs was obtained by affinity chromatography with *S*-hexylglutathione–Sepharose 6B as matrix [14]. GST isoenzymes were separated by cationic-exchange chromatography [CM-Sepharose 6B (fast flow); Pharmacia], resulting in two forms of electrophoretically pure GST 1-1, both with a specific activity towards 1-chloro-2,4-dinitrobenzene of 30 μmol/min per mg of protein at 25 °C. H.p.l.c. analysis of these isoenzymes, performed as described previously [15], illustrated their purity in terms of absence of other GST subunits. The first and major fraction showed two h.p.l.c. peaks, both

eluted after approx. 25 min. This set of peaks is usually termed 1–1, and is assumed to represent the two microheterologous forms of which distinct cDNA clones are known [16,17] (Fig. 1*a*). Unless otherwise stated, this fraction is used in this study. The second fraction consisted of only the later-eluted peak (Fig. 1*b*).

Inhibition assay

For all compounds tested, a standardized assay was applied: 0.5 ml of a 0.5 μM solution of enzyme (as calculated from its subunit concentration, by using an M_r of 25000, in 0.1 M-potassium phosphate buffer, pH 6.5, containing 1 mM-EDTA) was incubated with 5 μM-inhibitor at 0 °C. The incubation was started by adding the inhibitor and mixing the incubation mixture. At various time intervals between 5 and 400 s, 50 μl samples (i.e. 25 pmol of enzyme) were transferred to a cuvette containing 0.8 ml of the same buffer and 0.1 ml of 10 mM-GSH solution. The GSH is necessary for both the enzymic reaction and the termination of the inhibition process by reaction with the remaining inhibitor. After addition of 0.1 ml of a 10 mM solution of 1-chloro-2,4-dinitrobenzene in 40 % (v/v) ethanol, the conjugation was measured at 25 °C and 340 nm [18]. The time course of inhibition was analysed by computerized bi-exponential non-linear regression (smallest residual sum of squares, BMDP statistical software). All curves were composed of at least 24 data points, derived from three independent assays.

Quantification of the number of cysteine residues modified

The number of cysteine residues modified by quinones during the inactivation reaction was quantified by determination of the residual number of cysteine residues after the reaction with the quinone. This was achieved by incubation of the inhibited enzyme with a radiolabelled compound known to react quantitatively with thiol functions, i.e. tetraClBQ [8]. Complete reaction was achieved by incubation of the modified enzyme for 10 min at room temperature with a 10-fold molar excess of tetraCl[^{14}C]BQ. After this incubation, the enzyme was washed three times in a Centricon 10 micro-concentration device (Amicon, Danvers, MA, U.S.A.). The filter was dissolved in Soluene 350 (Packard) and the amount of radioactivity was determined by liquid-scintillation counting. With GST 1–1 that had not been incubated with quinones, this procedure resulted in modification of two residues per subunit.

Other procedures

$E_{\frac{1}{2}}$ values were determined for all quinones and their GSH conjugates by cyclic voltametry (scan rate 100 mV/s, maximal potential range +600 mV to −1000 mV) and differential pulse voltametry (scan rate 20 mV/s, modulation amplitude 25 mV, modulation time 5 ms, maximal potential range +400 mV to −1000 mV), with Ag/AgCl as reference electrode. The measurements were performed in the same buffer as used for the inhibition assay, at room temperature. The redox potentials of all quinones are presented in Table 1. For a number of quinones two $E_{\frac{1}{2}}$ values were observed. The lower values always showed relatively small currents as compared with the $E_{\frac{1}{2}}$ with the highest potential. All measurements were confirmed by differential pulse voltametry.

RESULTS

Synthesis and characteristics of conjugates

The mono-GSH conjugates of 1,4-benzoquinones and all chlorinated 1,4-benzoquinones, except for 2-Cl-3-GSBQ and 2-Cl-5-GSBQ, were synthesized and purified. Furthermore, the three mono-GSH conjugates of bromo-1,4-benzoquinone were synthesized and purified. Two types of reactions were observed. On the one hand, tetra-, tri- and two of the three dichlorobenzoquinones (the 2,5- and 2,3-isomers) reacted with GSH in a Michael-type 'addition–elimination' reaction, dechlorinating the quinone and leaving the product in the oxidized form. On the other hand, benzoquinone, chlorobenzoquinone and bromobenzoquinone reacted by a Michael addition, resulting in a hydroquinone conjugate. The 2,6-dichlorobenzoquinone produced both the dichlorinated (Michael addition) and monochlorinated (Michael-type 'addition–elimination') conjugates.

Inhibition of GST 1–1: structure–activity relationships

The covalent and irreversible inhibition of GST 1–1 by quinones was studied by measuring the time course of inactivation of the enzyme. The incubation of the enzyme and quinones was performed at 0 °C, since higher temperatures accelerated the process of inhibition to rates that could not be measured in the experimental set-up, especially for the tri- and tetra-chlorinated quinones and their conjugates.

For all quinones, inhibition was the result of covalent modification of the transferase isoenzyme. The maximal inhibition that could be reached was 90 %. For most compounds, this was reached within the experimental period, or after prolonged (10 min) incubation at 25 °C. If complete inactivation was not reached this way, subsequent addition of a 10-fold molar excess of tetrachloro-1,4-benzoquinone decreased the activity to approx. 10 % of the original activity (results not shown).

Fig. 2 presents the time course of inhibition of GST 1–1 with 2,5-diCl-3-GSBQ and 2,6-DiCl-3-GSBQ as reagents. The curves thus obtained for all quinones and their GSH conjugates were quantitatively analysed. Three methods were used to compare the inhibition characteristics. Table 2 presents the initial rates of inhibition, together with the time necessary to reach 50 % inhibition. Table 3 presents the results of non-linear-regression analysis of the experimentally obtained curves. The curves can be described as bi-exponential functions of time (eqn. 1), derived from non-linear regression analysis of the measured time course of inhibition (Table 3):

$$A_t/A_0 = a \cdot e^{-k_1 \cdot t} + (0.9 - a) \cdot e^{-k_2 \cdot t} + 0.1 \quad (1)$$

The two rate constants thus obtained are termed k_1 and k_2, and a determines the relative contributions of the two components to the inhibition. Since for all quinones the ultimate inhibition was approx. 10 % (i.e. the remaining activity after complete modification), the constant was assigned as 0.1. For a number of quinones a mono-exponential fit proved to be just as accurate. Apparently, two separate processes may be involved in the inhibition of GST 1–1. In order to ascertain that this biphasic effect was not due to the fact that the GST 1–1 enzyme preparation consisted of two heterologous forms (as shown in Fig. 1*a*) [16,17], a comparison of the heterologous and one of the homologous forms (see Fig. 1*b*) was made. For these two preparations, the time course of inhibition with 2,5-diClGSBQ was measured. No differences were observed in terms of the kinetic constants as presented in Tables 2 and 3. The remaining activity for both enzyme preparations was 10 %.

A number of observations are noteworthy: (i) increasing the number of chlorine atoms increased the rate of inhibition towards GST; (ii) the GSH moiety increased the rate of inhibition of the enzyme by the quinone; (iii) minor but interesting differences were observed between isomers. These observations are described in detail below.

(i) Effect of the halogen substituents. With the number of chlorine atoms increasing from zero to four, the rate of inhibition increased dramatically. Unsubstituted 1,4-benzoquinone does

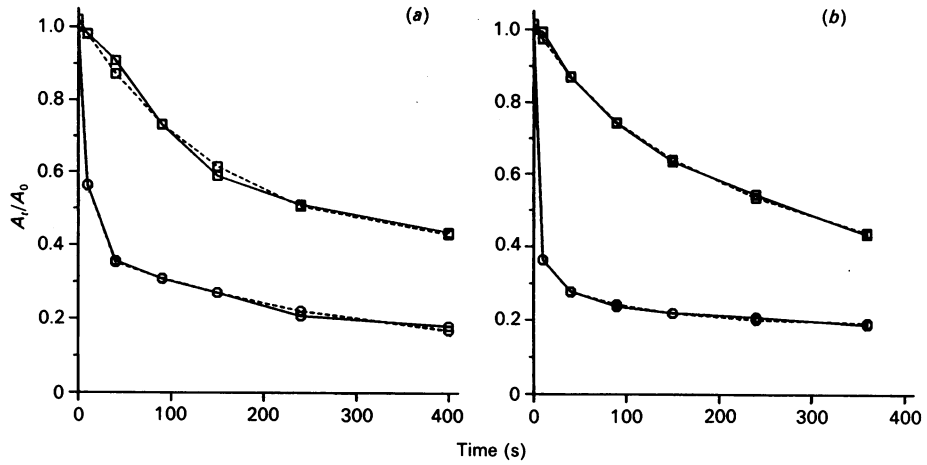

Fig. 2. Time course of inhibition obf GST 1–1 by 2,5-diClBQ (□) and its glutathione conjugate (○) (*a*) and by 2,6-diClBQ (□) and its GSH conjugate (○) (*b*)

Both the experimentally obtained curve (continuous line) and the curve fitted to a bi-exponential function (broken line) are presented. A_t/A_0 represents the enzyme activity (at time t s) divided by the initial activity ($t = 0$). Data points shown are averages of three independent incubations.

Table 2. Inhibition of GST 1–1 by halogenated quinones and their GSH conjugates

The time curve of inhibition of GST 1–1 by various quinones was determined by incubation of 0.5 μM enzyme (subunit concn.) with 5 μM inhibitor at 0 °C in a 0.5 ml volume. At various time intervals, 25 pmol enzyme samples were transferred to a cuvette containing 1.0 mM-glutathione, thus terminating the inhibition, and the remaining enzyme activity was determined. $\Delta A/\Delta t$ represents the initial rate of inhibition at $t = 0$, with A being the fractional activity (with value 1 for native enzyme), and $t(I_{50})$ represents the time (s) necessary to reach 50% inhibition.

Inhibitor	$\Delta A/\Delta t$ (s^{-1})	$t(I_{50})$ (s)	Targeting effect
BQ	0	∞	> 300
GSBQ	−0.0004	> 300	–
ClBQ	−0.001	> 300	–
6-Cl-2-GSBQ	−0.0004	> 300	–
2,3-diClBQ	−0.005	150	2
2,3-diCl-5-GSBQ	−0.041	75	
2,5-diClBQ	−0.004	260	13
2,5-diCl-3-GSBQ	−0.075	20	
2,6-DiClBQ	−0.004	290	41
2,6-diCl-3-GSBQ	−0.15	7	
TriClBQ	−0.34*	10	–
TriClGSBQ	−0.34*	7	
TetraClBQ	−0.20	5	
BrBQ	−0.002	> 300	
2-Br-3-GSBQ	−0.001	> 300	
2-Br-5-GSBQ	−0.0007	> 300	
2-Br-6-GSBQ	−0.0007	> 300	
TetraBrBQ	−0.19	9	

* Upper limit of detection.

Table 3. Inhibition of GST 1–1 by halogenated quinones and their GSH conjugates

GST 1–1 was inactivated by a series of quinones and their GSH conjugates as described in Table 2. The inhibition curve was fitted to the bi-exponential semi-logarithmic expression:

$$A_t/A_0 = a \cdot e^{-k_1 \cdot t} + (0.9 - a) \cdot e^{-k_2 \cdot t} + 0.1$$

with A representing the enzyme activity as a function of time, and k_1 and k_2 representing the rate constants of the two components of the inhibition reaction. The factor a determines the relative contributions of the two exponents to the inhibition.

Compound	a	k_1 (s^{-1})	k_2 (s^{-1})
BQ	No inhibition		
GSBQ	0.9	−0.0008	
ClBQ	0.9	−0.0006	
ClGSBQ	0.9	−0.0008	
2,3-DiClBQ	0.9	−0.0041	
2,3-DiCl-5-GSBQ	0.44	−0.0008	−0.08
2,5-DiClBQ	0.9	−0.0033	
2,5-DiCl-3-GSBQ	0.29	−0.0045	−0.12
2,6-DiClBQ	0.58	−0.0016	−0.001
2,6-DiCl-3-GSBQ	0.18	−0.0022	−0.21
TriClBQ	0.11	−0.0033	−0.37
TriClGSBQ	0.11	−0.0033	−0.37
TetraClBQ	0.23	−0.0011	−0.30
BrBQ	0.9	−0.0013	
Br-3-GSBQ	0.81	−0.0011	−0.09
Br-5-GSBQ	0.9	−0.0014	
Br-6-GSBQ	0.9	−0.0010	
TetraBrBQ	0.15	−0.013	−0.12

not cause any inhibition at all. For the mono- and di-chlorobenzoquinones, the rate of inhibition can largely be described by a single rate constant. Only for 2,6-dichlorobenzoquinone is a minor contribution of k_2 observed (Table 3). For tri- and tetra-chlorobenzoquinone, the rate of inhibition is largely determined by the fast component (k_2), with 80% inhibition already obtained after approx. 10s of incubation. For the bromobenzoquinones the time courses of inhibition of benzoquinones resemble those of their respective chlorinated counterparts.

(ii) Effect of the GSH moiety. For all quinones tested, addition of GSH to the ring structure increased the rate of inhibition (Tables 2 and 3). Although this effect is best seen with the dichlorinated benzoquinones, the inhibition is also stimulated for the quinones with one or no chlorine substituents. It is remarkable that, together with the introduction of the GSH

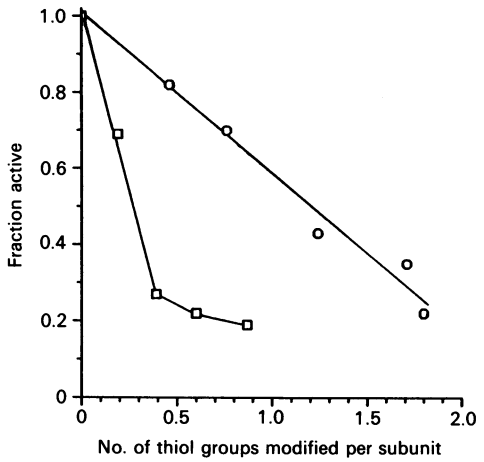

Fig. 3. Inhibition of GST 1–1 by 2,5-diClBQ (○) and its GSH conjugate (□), presented as a function of the amount of cysteine residues modified

See the Methods section for details of the experiment.

moiety to the dichlorinated benzoquinones, the second (k_2) process in the mathematical-fit model becomes of major importance. For the trichlorinated benzoquinone the rate of inhibition reached the limit of detection, thus masking possible stimulatory effects of the GSH moiety. It is remarkable that k_1 is only slightly affected by addition of GSH. The increase in rate of inhibition is largely due to both an increase in k_2 and the relative importance of this component.

The targeting action of the GSH moiety can be expressed as the ratio between the initial velocities of the quinone and its appropriate conjugate, by the ratios of the k_2 values or by the ratio of the times necessary to reach 50% inhibition. The largest targeting effect was observed for 2,6-dichlorobenzoquinone and its GSH conjugate (41, as calculated from '$t_{\frac{1}{2}}$' values). For a number of quinones this calculation was not possible, because of either a too high or a too low reactivity.

(iii) Differences between isomers. Two series of isomers have been assayed for their inhibitory action towards rat GST 1–1, i.e. the dichlorinated and monobrominated benzoquinones (Table 2). The GSH conjugates of bromobenzoquinone showed no differences in the inhibition of GST 1–1. As for the dichlorinated benzoquinones, firstly, the rate of inhibition increases in the order 2,6-isomer, 2,3-isomer and 2,5-isomer. Secondly, for the GSH conjugates a marked difference in rate of inhibition exists between the 2,3-isomer and the 2,6-isomer. Combining these two observations, the targeting effect ranges from 2 for the 2,3-isomers to 41 for 2,6-dichlorobenzoquinone and its conjugate. The 2,5-isomers show an intermediate value of 13 (Table 2).

Inhibition as a function of the benzoquinone concentration

In order to understand the physiological significance of the observed bi-exponential mode of inhibition for a number of compounds tested, the inhibition of rat GST 1–1 was measured as a function of the amount of benzoquinone bound to the protein. The amount of covalently modified cysteine residues was quantified by reaction of tetrachloro[^{14}C]benzoquinone with the cysteine residues that were not modified during the incubation with the inhibitors. With 2,5-dichlorobenzoquinone a linear relationship was observed between loss of catalytic activity and number of cysteine residues modified, with a maximum of two cysteine residues modified (Fig. 3). Thus with this compound 50% inhibition was achieved after modification of 50% of the cysteine residues present in the protein. However, with the corresponding GSH conjugate, modification of 50% of the cysteine residues already resulted in almost complete inhibition (Fig. 3). The same type of experiments with trichlorobenzoquinone and its GSH conjugate resulted in complete loss of activity after modification of only one cysteine residue for both the quinone and the conjugate (results not shown).

DISCUSSION

Chlorinated 1,4-benzoquinones have been shown to inactivate GSTs by covalent modification of a cysteine residue in or close to the active site [8,10]. Quinones are relatively reactive compounds and are described as toxic or as mediators of toxicity for many xenobiotics [19–21]. Optimizing their efficiency by means of targeting may be a useful tool in overcoming these toxicity problems and may allow the use of these compounds *in vitro* and *in vivo*. In the present study a quantitative structure–inhibition relationship has been established for quinone-mediated inactivation of GST 1–1.

Rat GST 4–4 appeared to be completely inhibited after chemical modification of one of the four cysteine residues [8]. The rat GST subunit of 1–1 contains two cysteine residues. By analogy with GST 4–4, modification of one of the two cysteine residues has a major impact on the enzyme activity. This cysteine residue is preferentially modified by 2,5-diCl-3-GSBQ, whereas the non-GSH-substituted parent 2,5-diClBQ reacts equally with both residues. The fact that GST 1–1 could always be inactivated to a maximal extent of 10% of the original enzyme activity, either by the direct action of the inhibitor or by subsequent reaction with tetraClBQ, suggests a common mechanism for all quinones: modification of cysteine residues of the enzyme results in stoichiometric inhibition for all quinones.

Complete modification of the cysteine residues never resulted in complete inactivation. This supports the theory that inactivation is not due to modification of a residue involved in the catalytic mechanism, but rather is a result of steric hindrance. This is also supported by the finding that completely modified GST isoenzymes have various residual activities with various substrates (results not shown).

The observed time course of inhibition of this isoenzyme suggests the involvement of two processes for the GSH conjugates and the tri- and tetra-chlorinated benzoquinones, whereas for the lower chlorinated benzoquinones a single process is involved. Quinones with affinity towards the active site (e.g. conjugates) preferentially modify the cysteine residue that contributes to the largest extent to the inhibition, i.e. the reaction described by rate constant k_2, whereas quinones with a low affinity for the active site will react at random with the two cysteine residues (no contribution of the second component). The results of the experiments with various benzoquinone concentrations support this theory: the GSH conjugate of 2,5-diClBQ inactivates the transferase completely at a 1:1 modification, whereas the benzoquinone itself has to react with both cysteine residues before it achieves complete inhibition. The reaction described by the k_1 function may be interpreted as a non-specific reaction with both cysteine residues. For most quinones only a minor change in k_1 is observed as a result of addition of GSH to the benzoquinone. This is most probably due to the fact that the chemical reactivity of the quinones towards thiol moieties is probably not strongly affected by GSH addition.

Although no information is presented on the chemical reactivity of the quinones, this parameter probably accounts for the increasing rates of inhibition observed with increasing number of chlorine atoms. Furthermore, the increasing lipophilicity of the compounds, resulting in an increasing affinity for the second substrate-binding site, may also contribute to the increase in

rates of inhibition. This is illustrated by the observation that trichlorobenzoquinone, in contrast with 2,5-dichlorobenzoquinone, completely inactivates the enzyme after modification of the first (active-site-located) of the two cysteine residues present. Tri- and tetra-chlorobenzoquinone are the only quinones for which the time course of inhibition is largely described by the affinity (k_2) component of the equation. For the chlorinated benzoquinones, the relation between the quinone/enzyme ratio and the inactivation has been reported previously [10,11].

The relative contribution of the two processes to the inhibition is described by the parameter a. The contribution to the inhibition of the affinity part of the equation may be as much as 88% (for trichlorobenzoquinone and its GSH conjugate). This also implies a contribution to the inhibition due to the modification of the cysteine residue close to the active site of at least 78%.

The targeting effect, as expressed by the ratio of inhibiting velocity between the conjugate and the quinone, does not require the description as a biphasic process: ratios of initial velocities or time necessary to reach 50% inhibition describe the targeting contribution of the GSH moiety in a way comparable with the ratio of the rate constants of the conjugate and the quinone. For the compounds that need two reaction rates to describe the inhibition, the k_1 constant is of relatively minor importance in the initial phase of inhibition.

The lack of correlation between rate of inactivation and the redox potential of the quinones tested suggests that no redox-related reactions, e.g. oxidation of cysteine thiol functions due to redox-cycling activity of the quinones, are involved in the process of inactivation. However, this mechanism cannot be excluded only on the basis of the overall redox potentials, since the stability of the semiquinone is of importance in this respect [22].

In conclusion a large number of halogenated quinones with their GSH conjugates have been evaluated for their inhibitory action towards GST 1–1. An increase in rate of inhibition was observed with increasing number of chlorine substituents. The most pronounced targeting effect, as observed for the 2,6-dichlorobenzoquinone (a 41-fold increase in rate of inhibition for the conjugate as compared with the quinone), is a promising starting point in terms of application to more complex systems (intact cells), especially since the quinone involved is only moderately reactive towards thiol compounds.

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