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INTERACTION OF EBSELEN WITH GLUTATHIONE *S*-TRANSFERASE AND PAPAIN *IN VITRO*

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Abstract—The interaction of ebselen(2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) with rat liver cytosolic glutathione *S*-transferases (GSTs) and the plant cysteine protease, papain, was studied as cysteine residues are important for the activity of these enzymes. The capacity of GST 1-2 and 3-4 for ebselen binding is similar (1.5 mol ebselen/mol GST isozyme), while GST 2-2 and GST 7-7 bind 0.3 and more than 2.0 mol ebselen/mol GST isozyme, respectively. Ebselen does not bind to *N*-ethylmaleimide-treated GST, and its binding to GST is prevented by 5 mM thiols. Ebselen irreversibly inactivates the different GST isozymes with a second order rate constant ranging from 20 to 2250 M^{-1} sec^{-1} for the different subunits. GST inhibition by ebselen is partially restored by 5 mM thiols. Ebselen binds to untreated papain and to cysteine-treated papain at a ratio of about 0.1 and 0.75 mol ebselen/mol papain, respectively. Ebselen does not bind to *N*-ethylmaleimide-treated papain, and its binding to papain is interfered with by added thiols. Papain is inactivated by ebselen with a second order rate constant of 1800 M^{-1} sec^{-1} in the absence of thiols. However, in the presence of GSH, 2-mercaptoethanol or sodium borohydride, ebselen exerts an activating effect on papain. The binding of ebselen by a seleno-sulfide bond to cysteine residues of GSTs and papain leads to their inactivation.

Key words: selenoorganic compound; ebselen; thiols; glutathione; cysteine

Ebselen (PZ51; 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) is a selenium-containing heterocyclic compound, which exhibits GSH† peroxidase-like activity *in vitro* [1,2; see Ref. 3 for review]. In the presence of sulfhydryl groups the isoselenazole ring of ebselen is reductively opened and forms a seleno-sulfide linkage with the sulfhydryl groups of proteins [4]. This interaction of ebselen may be important for its metabolism and pharmacological action.

Cytosolic GSTs (EC 2.5.1.18), a family of multifunctional proteins, catalyse GSH conjugation with electrophilic compounds [5, 6]. GSTs serve in the intracellular detoxication of chemical substances and play a role as an intracellular carrier protein (ligandin) of organic molecules similar to albumin in blood plasma. The amount of cysteine residues varies among the different GST subunits (2 cysteine/mol GST 1 subunit [7, 8]; 1 cysteine/mol GST 2 subunit [9]; 3 cysteine/mol GST 3 subunit [10]; 3 cysteine/mol GST 4 subunit [11]; 4 cysteine/mol GST 7 subunit [12]). Though the active centers of GST isozymes contain no cysteine, the modification of this amino acid affects enzyme activity [13].

In contrast to GST, papain (EC 3.4.22.2), a cysteine protease isolated from *Papaya latex* [14], contains one cysteine residue in the active center, essential for catalytic activity. Like all cysteine proteases, papain is activated by thiols and inactivated by thiol-blocking reagents [15].

Here, we studied the interaction of ebselen with the sulfhydryl groups of GST and papain and its effect on enzyme activity.

MATERIALS AND METHODS

Materials. Ebselen and [^{14}C]ebselen (3.7×10^{11} Bq/mol) were a kind gift from Rhône-Poulenc-Nattermann (Cologne, F.R.G.). Sephadex G25 and G-75 were from Pharmacia (Uppsala, Sweden). Dynamax-300A C18 was from Rainin (Woburn, U.S.A.). Papain, *N*-benzyloxycarbonyl-phenylalanyl - arginine - 4-methyl-coumaryl-7-amide (*Z*-Phe-Arg-*NH*-Mec) and CDNB were from Sigma (Deisenhofen, F.R.G.). *N*-Ethylmaleimide was from Merck (Darmstadt, F.R.G.). All other reagents were standard analytical grade products.

Preparation of GST. Cytosolic GSTs (unfractionated GSTs) were isolated from male Wistar rat liver according to Meyer *et al.* [16]. Purification of GST isozymes was carried out according to Mannervik and Jensson [17] with some modification. GST isozymes were identified on the basis of their retention times on reverse-phase HPLC [16]. Unfractionated GSTs purified from rat liver contained mainly the subunits 1, 2, 3 and 4 at a molar ratio of 1:1.4:1.2:1.8, respectively. GST 7-7 was isolated from rat Zajdela hepatoma cells (Deutsches Krebsforschungszentrum, Heidelberg, F.R.G.) by GSH-Sepharose affinity chromatography.

Assay of enzyme activity. GSH conjugation activity of GST with CDNB (1 mM) and GSH (1 mM) as

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† Abbreviations: GST, glutathione *S*-transferase; GSH, glutathione; GSSG, glutathione disulfide; CDNB, 1-chloro-2,4-dinitrobenzene; Z, *N*-benzyloxycarbonyl; Mec, 4-methyl-coumaryl-7-amide.

substrates was determined as described by Habig *et al.* [18]. One unit of GST activity was defined as that conjugating 1 μmol CDNB/min at 25°. In the inhibition studies, GST was pre-incubated with ebselen for the indicated times and then enzyme activity was measured. From the apparent first-order rate constants derived from the inactivation plots, the second-order rate constants were calculated by division with ebselen concentration [19]. Protease activity of papain was measured according to the method of Barrett and Kirschke [20]. One unit of protease activity was defined as that degrading 1 μmol *Z*-Phe-Arg-*NH*-Mec/min at 37°. When the second-order rate constants of ebselen for papain were calculated, papain activity was measured in the absence of thiols. Protein concentration was measured by the method of Bradford [21] with BSA as standard.

Binding of ebselen to GST and papain. Unfractionated GSTs (3.3 μM, based on an assumed average M_r of 50,000 for the dimer), the different GST isozymes (3.3 μM) and papain (3.3 μM) were incubated with [^{14}C]ebselen at 37° for 10 min in 100 mM potassium phosphate buffer, pH 6.5, with or without thiols, such as 2-mercaptoethanol, cysteine and dithiothreitol. After trichloroacetate precipitation, GST and papain were collected on glass fiber filters and unbound ebselen was washed out with ethyl acetate. The amount of ebselen bound to enzymes was calculated by radioactivity and protein concentration.

To examine the binding to activated papain, papain was treated with 10 mM cysteine at 0° for 2 hr. Residual cysteine was eliminated by fractionation on a Sephadex G-25 column (0.5 × 5 cm). The amount of ebselen binding to activated papain was determined in the same way as described above. The effect of *N*-ethylmaleimide on ebselen binding to GST or papain was also investigated. Unfractionated GSTs or papain were incubated with 1 mM *N*-ethylmaleimide at 37° for 15 min. Unbound *N*-ethylmaleimide was eliminated on the same Sephadex G-25 column. The amount of ebselen binding to these *N*-ethylmaleimide-treated enzymes was determined.

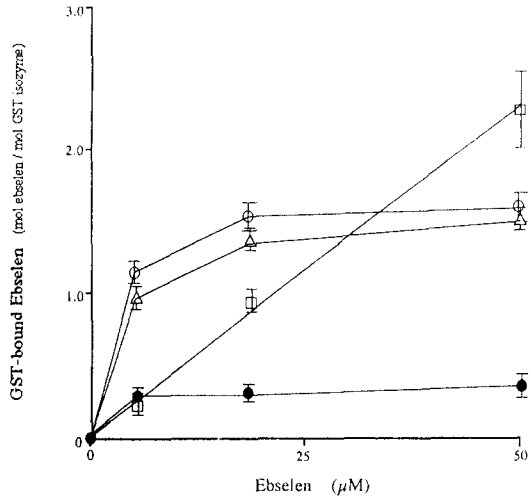


Fig. 1. Ebselen bound to different GST isozymes. The amount of ebselen bound to different GST isozymes (3.3 μM) was determined as described in Materials and Methods. Values are the means ± SEM of four determinations. GST 1-2 (○), GST 2-2 (●), GST 3-4 (△), GST 7-7 (□).

Change in the UV spectrum of ebselen upon incubation with GST 7-7. Ebselen (20 μM) was incubated with increasing concentrations of GST 7-7 (0–20 μM) in 10 mM potassium phosphate buffer, pH 7.5, containing 0.2% dimethylformamide for 10 min at 25°. The spectrum was monitored in a UV spectrometer (Perkin–Elmer Lambda 2).

RESULTS

Binding of ebselen to GST isozymes

Ten minutes after incubation of GST isozymes of the different classes (3.3 μM dimeric enzyme) with increasing ebselen concentrations, GST 1-2 and 3-4 were saturated at about 10 μM ebselen (3-fold molar excess) (Fig. 1). The amounts of ebselen bound to

Table 1. Effect of thiols and *N*-ethylmaleimide on the binding of ebselen to GSTs

	mM	Binding of ebselen (mol ebselen/mol subunit)	(% of control)
Unfractionated GST only	Control	1.08	100
+ mercaptoethanol	0.1	0.98	91
	5.0	0.37	34
+ dithiothreitol	0.1	0.63	58
	5.0	0.24	22
+ cysteine	0.1	0.90	83
	5.0	0.22	20
+ GSH	0.1	1.10	100
	5.0	0.12	11
N-Ethylmaleimide-treated GSTs		0.22	20

Unfractionated GSTs (3.3 μM) were incubated with a 10-fold molar excess of ebselen in the presence of thiols (0.1 or 5 mM) at 25° for 10 min. The same amount of *N*-ethylmaleimide-treated GSTs were also incubated with a 10-fold molar excess of ebselen.

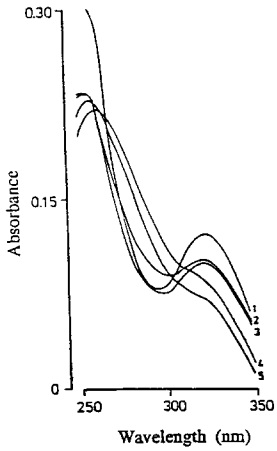


Fig. 2. Change in the UV spectrum of ebselen upon interaction with GST 7-7. Ebselen (20 μM) was incubated with GST 7-7 at the indicated concentrations. The spectrum of ebselen was obtained as described in Materials and Methods. 1: 0 μM GST 7-7 (control); 2: 0.3 μM; 3: 1.3 μM; 4: 5.0 μM; 5: 20 μM.

GST 1-2 (3 cysteine residues/mol) and 3-4 (6 cysteine residues/mol) were similar, being about 1.5 mol ebselen/mol isozyme. In contrast, under identical conditions GST 2-2 (2 cysteine residues/mol) bound only about 0.3 mol ebselen/mol isozyme. The amount of ebselen bound to GST 7-7 (8 cysteine residues/mol) increased linearly with ebselen concentration, up to 50 μM ebselen (15-fold molar excess) (Fig. 1).

Effect of thiols and N*-ethylmaleimide on ebselen binding to GST*

In the presence of 0.1 mM thiols as 2-mercaptoethanol, dithiothreitol, cysteine or GSH, the binding of ebselen to GSTs was hardly affected, but at 5 mM thiols it decreased to about 10–35% of the control (Table 1). When 5 mM thiols were added after interaction of ebselen with GSTs, they released ebselen from its GST complex (data not shown). Furthermore, the amount of ebselen binding to *N*-ethylmaleimide-treated GSTs decreased to 20% of control (Table 1). These findings suggest that ebselen interacts with cysteine residues of GSTs.

Change of the UV spectrum of ebselen upon interaction with GST 7-7

After incubation of ebselen with GST 7-7, an enzyme concentration-dependent change in the UV spectrum of ebselen was observed (Fig. 2). The maximum/minimum ratio A_{323} to A_{295} of about 1.75 (no GST) was decreased to 0.63 (20 μM GST). This finding indicates that the selenazole ring of ebselen was opened [22]. A similar effect was measured with BSA and the thiols GSH and cysteine. When ebselen was incubated with *N*-ethylmaleimide-treated GST, the spectra were unaffected (data not shown). Incubation of ebselen with 1 mM sodium borohydride had no effect on the spectrum.

Table 2. Inactivation of the different GST isozymes and papain by ebselen

Enzyme	Second-order rate constant (M^{-1} sec^{-1})
GST	
1-1	2250
1-2	150
2-2	20
3-3	1600
3-4	940
4-4	2100
7-7	1160
Papain	1800

The second-order rate constants were determined as described in Materials and Methods.

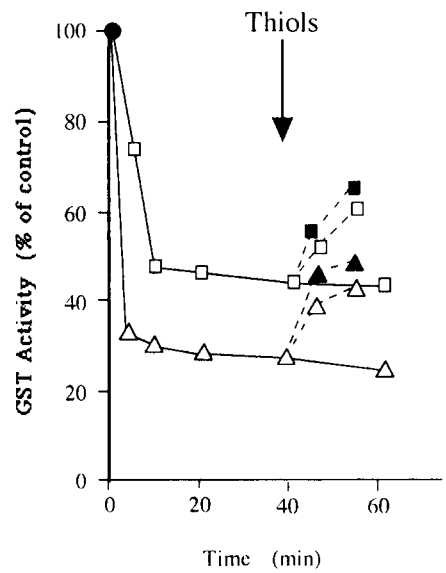


Fig. 3. Time course of inhibition of GST activity by ebselen. Unfractionated GSTs (9 μM) were incubated with 50 μM ebselen (□) or 100 μM ebselen (△) at 37° in 100 mM potassium phosphate buffer, pH 6.5, and GST activity was measured at the indicated times. After 40 min incubation, 5 mM 2-mercaptoethanol (△, □) or 5 mM dithiothreitol (▲, ■) was added (dotted lines). The GST activity without ebselen (control) exhibits the same value (20.3 ± 2.3 U/mg protein) at the indicated times.

Inhibition of GST by ebselen

All GST isozymes tested were irreversibly inhibited by ebselen, because activity could be restored only after reduction with thiols. As summarized in Table 2, the second-order rate constants of ebselen were in the range 940–2250 M^{-1} sec^{-1} for all isozymes with the exception of the enzymes containing subunit 2 with low second-order rate constants (20 or 150 M^{-1} sec^{-1}).

The time-dependent inactivation of unfractionated GSTs by ebselen is shown in Fig. 3. The activity of

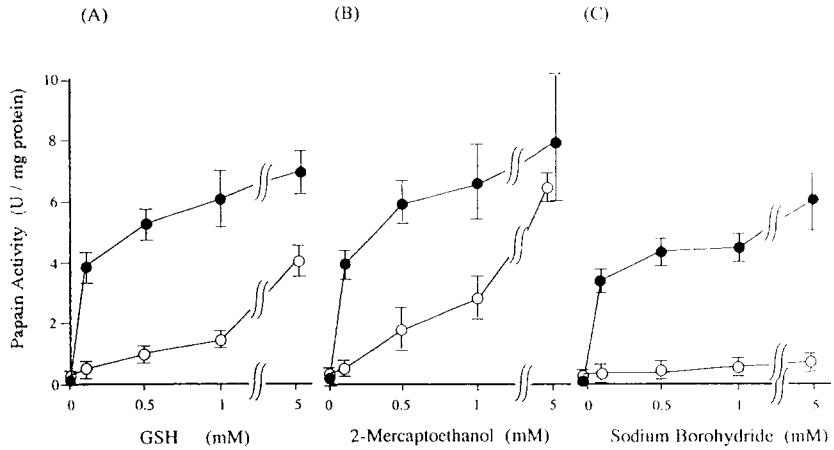


Fig. 4. Effect of ebselen and reductants on papain activity. Papain (5 μM) was incubated with ebselen [(○) 0 μM, (●) 20 μM] and reductants [(A) GSH, (B) 2-mercaptoethanol, (C) sodium borohydride] at indicated concentrations for 15 min at 37° in 50 mM sodium phosphate buffer, pH 7.0. Values are means ± SEM of four determinations.

GST was decreased to about 50% and 30% of control by 50 and 100 μM ebselen, respectively, within the first 10 min. When 5 mM dithiothreitol or 5 mM 2-mercaptoethanol were added 40 min after incubation, the activity of GST inhibited by ebselen was partially restored.

Binding of ebselen to papain or N*-ethylmaleimide-treated papain*

When papain or cysteine-treated (activated) papain was incubated with a 10-fold molar excess of ebselen, ebselen bound to untreated papain and cysteine-treated papain at a ratio of 0.1 and 0.75 mol ebselen/mol papain, respectively. However, ebselen did not bind to papain in the presence of thiols, nor to *N*-ethylmaleimide-treated papain (data not shown).

Effect of ebselen on papain activity

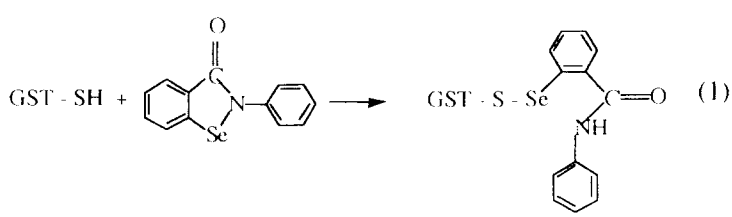
The specific activity of the papain tested was 0.1 ± 0.01 U/mg protein in the absence of reductants. Under this condition, papain was irreversibly inhibited by ebselen at a second-order rate constant of 1800 $M^{-1} sec^{-1}$ (Table 2). In the presence of reductants (0.1 mM) such as GSH, 2-mercaptoethanol and sodium borohydride, papain was activated (0.4 ± 0.1 U/mg protein). Under this condition, however, an unexpected effect of ebselen on papain was observed, in that the specific activity of papain was increased about 10-fold by 20 μM ebselen (3.8 ± 0.6 U/mg protein), as shown in Fig. 4. The possibility that some components exhibited the artificial activity is excluded. This activation was independent of the sequence of incubation, and it was not observed when GSSG was used instead of GSH (data not shown).

DISCUSSION

Interaction of ebselen with GST

We have demonstrated that ebselen binds to GST and that its binding leads to inactivation. Thiols interfere with the binding of ebselen to GST and release ebselen from ebselen–GST complexes. GST inhibition by ebselen is restored by thiols. Ebselen does not bind to GST treated with the SH reactant, *N*-ethylmaleimide. Furthermore, the selenazole ring of ebselen is opened after incubation with GST 7-7. A similar effect is observed with other compounds containing thiol groups, such as BSA, GSH and cysteine. It is concluded that the selenazole ring of ebselen is reductively opened by SH groups of GST resulting in the cleavage of the Se-N bond followed by the formation of the Se-S bond between ebselen and GST, as shown in reaction (1).

Ebselen inhibited GST irreversibly. However,



from the data on the inactivation of GST by ebselen (Fig. 3), it appears that about 20% GST activity still remained. This may be due to the assay conditions used to measure GST activity where 1 mM GSH was added as substrate. This may have partially reactivated the enzyme.

Role of cysteine residues for catalytic activity of GST

GSTs of the π class (GST 7) share susceptibility for SH reagents. It has been reported that SH modification of cysteine-47 of GST 7 with *N*-ethylmaleimide [23], GSSG [24], hydrogen peroxide [25] and CDNB [26] results in inactivation. Shen *et al.* [13] reported that cysteine-101 as well as cysteine-47 are located in a region important for GSH binding and that the disulfide bond formation between these residues results in steric hindrance. Recently, the three-dimensional X-ray analysis of the porcine GST 7 [27] and the rat GST 3 [28, 29] clearly ruled out a direct participation of cysteine residues in catalysis. Therefore, it is considered that the modification of cysteine residues may result in conformational changes in GST. Our results also demonstrate that the modification of cysteine residues of the different rat liver GST isozymes by ebselen leads to enzyme inhibition.

The amount of ebselen bound to GST 2-2 is very low (about 0.3 μM ebselen/mol isozyme) and the second-order rate constant of ebselen for GST 2-2 is much less than those for other GST isozymes. GST 2 contains only 1 cysteine residue/mol subunit. This cysteine residue (cysteine-211; total of 220 amino acid residues) is located in the C-terminal region, and it may be sterically protected against ebselen modification. In contrast, the amount of ebselen bound to GST 7-7 increases linearly with the ebselen concentration, determined up to 50 μM ebselen (15-fold molar excess). It is concluded that this saturation is SH-group dependent, because GST 7-7 with eight SH-groups per isozyme [12] has the highest SH-group content.

Interaction of ebselen with papain

Papain contains 7 mol cysteine/mol papain, present as three disulfide linkages and one free sulfhydryl group in the active center, essential for protease activity. It is partially masked in the absence of thiols, because it can be detected after treatment with thiols [30], and cysteine proteases including papain are activated by sufficient thiols [16]. As shown in this paper, the amount of ebselen binding to cysteine-treated (activated) papain is 7.5-fold greater than that to untreated papain. In the absence of thiols, papain is completely inactivated by ebselen. Furthermore, ebselen does not bind to papain treated with *N*-ethylmaleimide. These findings suggest that ebselen binds to the free sulfhydryl group of papain and inactivates it.

Unexpectedly, papain is activated by ebselen in the presence of reductants, such as GSH, 2-mercaptoethanol and sodium borohydride. The mechanism of this activation was not examined further in this study.

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