Biochemical Pharmacology, Vol. 37, No. 11, pp. 2267–2271, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Press plc

KINETIC MECHANISM AND SUBSTRATE SPECIFICITY OF GLUTATHIONE PEROXIDASE ACTIVITY OF EBSELEN (PZ51)

MATILDE MAIORINO, ANTONELLA ROVERI, MARIAGRAZIA COASSIN and FULVIO URSINI*
Department of Biological Chemistry of the University of Padova, v. Marzolo 3, 35121 Padova, Italy

(*Received* 23 *October* 1987; *accepted* 16 *December* 1987)

Abstract—The glutathione peroxidase activity of ebselen (PZ51) was studied using different hydroperoxidic substrates. The single progression curves obtained in the spectrophotometric test were processed by a computer to fit the integrated rate equation that describes the ping pong reaction of the Se glutathione peroxidases. Ebselen catalyzes the GSH peroxidase reaction with a mechanism that appears kinetically identical to the mechanism of the enzymes. The inactivation of the catalytic properties of ebselen by iodoacetate suggests that a selenol moiety is involved. Among the substrates tested, the best hydroperoxidic substrates are the hydroperoxy derivatives of phosphatidyl choline. Ebselen is active also on membrane hydroperoxides as does phospholipid hydroperoxide glutathione peroxidase but not glutathione peroxidase.

Hydroperoxides that are produced in aerobically living cells, either by oxidative reactions or by specific enzymes, can be harmful to biological structures, owing to their susceptibility to homolytic or heterolytic cleavage, that leads to the formation of free radicals [1]. The most studied examples are the formation of hydroxyl [2] or alkoxyl [1] radicals ($HO^{\cdot}$, $RO^{\cdot}$) from hydrogen peroxide or a lipid hydroperoxide, respectively, by a Fenton-type reaction between the hydroperoxide and Fe^{2+}. The biological protection against these oxidizing radicals includes two lines of defence [3]: the antioxidants, or radical scavengers that quench free radicals and the peroxidases that prevent their formation by reducing the hydroperoxides to the corresponding harmless hydroxy derivatives. The removal of hydrogen peroxide is carried out by catalase, as well as by glutathione peroxidase [4] (GPX)† The organic hydroperoxides are reduced by GPX [5] as well as by glutathione S-transferase B (the so called Se-independent glutathione peroxidase) [6]. The membrane hydroperoxides (on which none of the previous enzymes is active) are reduced only by the Se-dependent phospholipid hydroperoxide glutathione peroxidase (PHGPX) [7]. These peroxidases, by keeping low the hydroperoxide concentration in different cellular compartments, protect the cells from oxidative damage. Moreover the hydroperoxide concentration, that depends on the balance between their synthesis and reduction, plays a key role on the activation of cycloxygenases [8] and lipoxygenases [9]. Therefore the metabolism of different hydroperoxides seems to be involved in cellular physiological and pathological responses, among which inflammation is the best known. Ebselen (PZ51) (Fig. 1) is a synthetic organoselenium compound with antiinflammatory activity [10], that exhibits a glutathione peroxidase-like activity [11]. This compound protects hepatocytes from lipid peroxidation induced by ADP-Fe, but it is ineffective in GSH depleted cells [12]. Moreover this compound inhibits the neutrophil lipoxygenase [13] and protects against galactosamine-endotoxin-induced hepatitis in mice, and this protection was interpreted in terms of an inhibition of the leukotriene pathway [14].

In the present work we compare the kinetic mechanism and the substrate specificity of ebselen with those of Se-dependent peroxidases. The kinetics of GPX was studied under steady state conditions analyzing either the initial rates of the reaction in the presence of different concentrations of hydrogen peroxide [15], or by computer processing of the single progression curves to fit the integrated rate equation [16]. In both cases the results were compatible with a tert-uni ping pong mechanism, where the selenol of the selenocysteine of the enzyme is first oxidized by the hydroperoxide to a selenenic acid which in turn is reduced back by two GSH. The same mechanism has been demonstrated for PHGPX [7].

$$\text{ROOH} + \text{E-Cys SeH} \xrightarrow{K_1} \text{E-Cys SeOH} + \text{ROH}$$

$$\text{E-Cys SeOH} + \text{GSH} \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} \text{E-Cys Se-SG} + \text{H}_2\text{O}$$

$$\text{E-Cys Se-SG} + \text{GSH} \underset{K_{-3}}{\overset{K_2}{\rightleftharpoons}} \text{E-Cys SeH} + \text{GSSG}$$

The present report provides information on the catalytic mechanism of the peroxidase activity of ebselen that did appear very similar to that of Se-

* To whom correspondence should be addressed.

† Abbreviations used: PZ51, 2-phenyl-1,2-benzo-isoselenazol-3(2H)one; GSH, reduced glutathione; GPX, glutathione peroxidase; PHGPX, phospholipid hydroperoxide glutathione peroxidase; DTE, dithiorythritol; LAHPO, linoleic acid hydroperoxide; PC-OOH, phosphatidyl choline hydroperoxide.

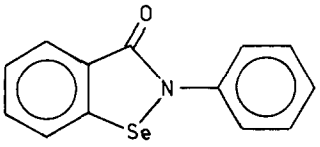

Fig. 1. Ebselen (PZ51).

dependent peroxidases. Moreover the substrate specificity of ebselen is more similar to that of PHGPX than to that of GPX.

MATERIALS AND METHODS

Ebselen was a gift of Dr E. Graf, Natterman Cie GmbH, Koln, F.R.G. Phosphatidyl choline hydroperoxides were prepared using soybean lipoxygenase and phosphatidyl choline, as described [17]. Linoleic acid hydroperoxide was prepared as described [7]. The kinetics of the peroxidase reaction was studied as described in Ref. 7. The digitalized readout of the absorbance at 340 nm of the reaction mixture containing, in 2.5 ml, 2.5–10 μM, 50–100 μM peroxidic substrate, 1–5 mM glutathione, 3 IU glutathione reductase, 5 mM EDTA and 0.15 mM NADPH in 0.15 M Tris–HCl buffer, pH 7.5, were recorded by an Apple IIe minicomputer. The reaction was carried out at 37° and a stirring equipped cuvette was used. The starting reagent was the hydroperoxide. Beers' law was followed up to an absorbance of 2.5. The computer allowed the rapid processing of the absorbance decrease data to fit the integrated rate equation [7, 16]. For the inactivation experiments, ebselen (125 μM) was incubated at room temperature with 0.5 mM iodoacetate and 0.5 mM thiol, in 50 mM Tris–HCl 0.1 mM EDTA, pH 7. At different times the peroxidase activity was measured on aliquots containing 6 nmoles of ebselen. The peroxidic substrates were 60 μM phosphatidyl choline hydroperoxides. Rat liver microsomes were prepared and peroxidized for 30 min as described [18]. The peroxidized microsomes were used as source of hydroperoxides for the peroxidase reaction of either ebselen or PHGPX. When peroxidized membranes were used as substrate the peroxidase activity test mixture was supplemented with 0.3% Triton X-100. PHGPX was prepared as described [7].

RESULTS

The kinetics of the peroxidase activity of ebselen was studied using the integrated rate equation that fits the experimental data obtained using either GPX [15] or PHGPX [7]. This approach was suggested by the evidence that ebselen is a catalyst of the glutathione peroxidase reaction. Moreover it was plausible to suspect that the data obtained using a simple chemical compound as catalyst could fit the kinetics of GPX and PHGPX, where a ping pong reaction takes place, without the formation of central complexes. The time course of GSH oxidation was recorded by measuring the NADPH oxidation in a reaction mixture containing the peroxidic substrate, and the GSH regenerating system. The absorbance was recorded until all hydroperoxides were consumed. The computer then processed the absorbance data to fit the integrated rate equation [16]:

$$\frac{[E] \times t}{[ROH]_t} = \frac{\phi_1 \ln \dfrac{[ROOH]}{[ROOH] - [ROH]_t}}{[ROH]_t} + \frac{\phi_2}{[GSH]}$$

The initial concentration of the hydroperoxide (ROOH) as well as the concentration of the reduced product (ROH) were calculated from NADPH absorbance. The concentration of GSH was held constant by GSSG reductase and NADPH.

The plot of $t/[ROH]_t$ versus $\ln([ROH]/[ROOH]-[ROH]_t)/[ROH]_t$ led to straight lines in the presence of all the peroxidic substrates used (hydrogen peroxide, cumene hydroperoxide, linoleic acid hydroperoxide and phosphatidyl choline hydroperoxides) and different GSH concentrations led to parallel lines. The plot obtained using phosphatidyl choline hydroperoxides as substrate is reported in Fig. 2.

The slope of these lines corresponded to $\phi_1/[E]$ and the intercept to $\phi_2/[E] \cdot 1/[GSH]$. The kinetic coefficients ϕ_1 and ϕ_2 (Table 1) were calculated from these values.

The kinetic behaviour of ebselen is therefore compatible with a tert-uni ping pong mechanism, identical to that reported for Se peroxidases [15, 16].

Because the kinetic coefficients are defined as follows

$$\phi_1 = 1/K_1 \text{ and } \phi_2 = 1/K_2 + 1/K_3$$

the rate constants for the interaction of ebselen with peroxidic substrates could be also calculated. The apparent second order rate constant for the interaction between ebselen and hydroperoxides are reported in Table 2, where the rate constant for the interaction between the hydroperoxides and the Se peroxidases are also reported for comparison.

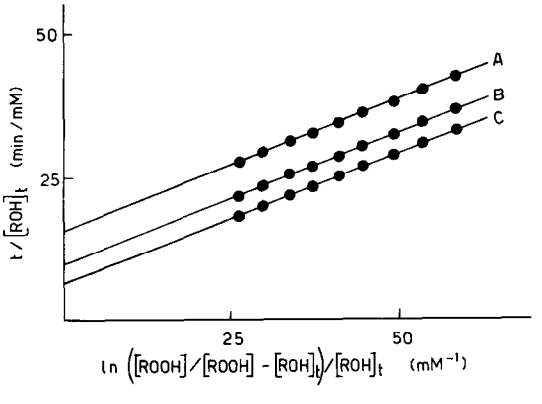

Fig. 2. Linear plots of the integrated rate equation for glutathione peroxidase reaction catalyzed by ebselen on phosphatidyl choline hydroperoxides. The analysis was carried out as described in the text, the ebselen concentration was 10 μM, the initial concentration of hydroperoxides was 60 μM, the concentration of GSH was 1 mM (A), 2 mM (B) or 3 mM (C).

Table 1. Dalziel coefficients (ϕ_1 and ϕ_2) for the glutathione peroxidase reaction of ebselen with different hydroperoxides

Substrate	ϕ_1 (mM min)	ϕ_2 (mM min)
Hydrogen peroxide	1.5×10^{-2}	6.6×10^{-1}
Cumene hydroperoxide	3.3×10^{-2}	1.4
Linoleic acid hydroperoxide	5.6×10^{-3}	3.5×10^{-1}
Phosphatidyl choline hydroperoxides	1.1×10^{-3}	7.3×10^{-2}

The kinetic coefficients were calculated from the plot of the integrated rate equation as described in the text. The GSH was 3 mM and the hydroperoxides 60 μM.

Table 2. Apparent second order rate constants (K_1) for the reaction between ebselen, GPX and PHGPX and hydroperoxidic substrates

Substrate	Ebselen	GPX $K_1(mM^{-1} min^{-1})$	PHGPX
Hydrogen peroxide	6.6×10^1	2.9×10^6	1.9×10^5
Cumene hydroperoxide	3.0×10^1	1.0×10^6	1.3×10^5
Linoleic acid hydroperoxide	1.8×10^2	1.8×10^6	2.3×10^6
Phosphatidyl choline hydroperoxides	8.8×10^2	—	7.0×10^5

The rate constant were calculated from the linear plots of the integrated rate equations, as described in the text. The GSH was 3 mM and the hydroperoxides 60 μM. The rate constants of GPX and PHGPX are from Refs 16 and 7.

In Se peroxidases, despite the lack of direct evidence of the two oxidation states of selenium, a redox shuttle between −SeH and −SeOH has been strongly suggested [5]. However, neither of these species is present in ebselen. Therefore an "activation" of the compound must occur and has been recently described [19]. The involvement in the peroxidatic reaction of a selenol is suggested by the inactivation experiments in the presence of iodoacetate. The peroxidase activity of ebselen is indeed inhibited when the compound is incubated in the presence of iodoacetate and reducing agents, with pseudo-first order kinetics (Fig. 3). An identical inhibition kinetics has been reported for GPX [16] and PHGPX [7]. This fast displacement of iodide requires a strong nucleophile such as the selenolate anion that is maintained in the reduced form by the thiols (GSH or DTE). In the absence of reducing agents, as well as in the presence of reducing agents that cannot reduce the active site (e.g. mercaptoethanol), the iodacetate fails to inactivate, while in the presence of cysteine the kinetics of the inactivation is very slow. These results suggest that DTE, but not the other thiols, can substitute for GSH in the peroxidatic reaction. This is in agreement with the previous observation that DTE can substitute for GSH in protecting, in the presence of ebselen, isolated hepatocytes from lipid peroxidation [12].

As shown in Table 2 phospholipid hydroperoxides, that are substrates for PHGPX but not for GPX [7],

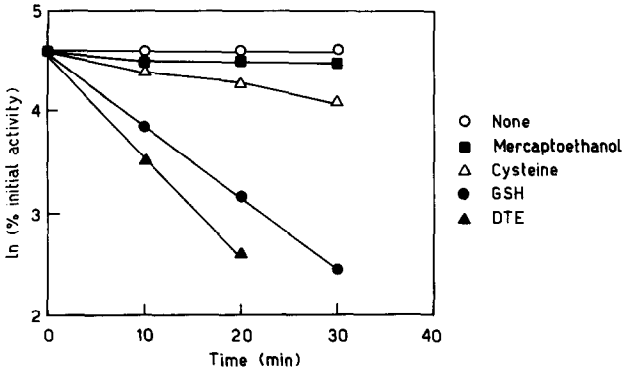

Fig. 3. Kinetics of inactivation of peroxidase activity of ebselen in the presence of iodoacetate. Ebselen (125 μM) was incubated at room temperature with 0.5 mM iodoacetate and 0.5 mM thiols, in 50 mM Tris–HCl, 0.1 mM EDTA, pH 7. At different times the peroxidase activity was measured on aliquots containing 6 nmoles of ebselen. The peroxidic substrate were 60 μM phospholipid hydroperoxides.

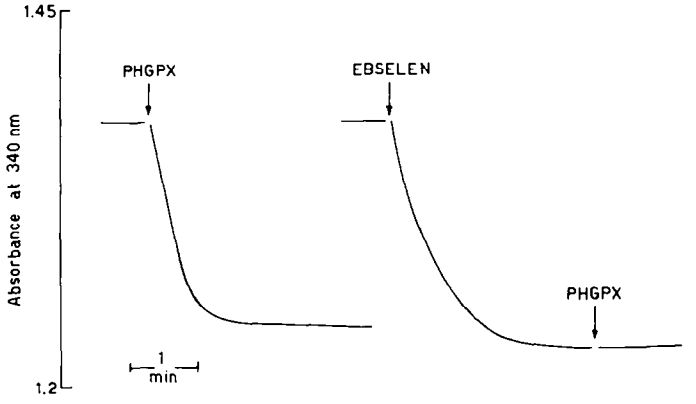

Fig. 4. Glutathione peroxidase activity of ebselen on membrane hydroperoxides. Peroxidized rat liver microsomes were used, following peroxidation, as source of peroxidic substrate for ebselen (30 μM) and for PHGPX (10 nM). The reaction mixture, contained 0.3% Triton X-100. The GSH was 3 mM. For details see Ref. 18.

are the best substrate for the peroxidase activity of ebselen. Due to this activity on phospholipid hydroperoxides, possibly linked to the lipophilicity, ebselen can also reduce the hydroperoxides in the membranes. The experiments reported in Fig. 4 show that ebselen reduces in peroxidized membranes all the hydroperoxides that are reduced by PHGPX and it has been previously reported that PHGPX reduces all the hydroperoxides in the membranes [18].

DISCUSSION

The kinetics of the glutathione peroxidase-like reaction of ebselen is in agreement with a tert-uni ping pong mechanism, apparently identical to the mechanism of selenium dependent peroxidases. This mechanism requires a redox shuttle of the selenium and the inactivation experiments in the presence of iodoacetate indicate the involvement of a selenol. In fact in the presence of the substrates ebselen undergoes modifications leading to the formation of the catalytically active form [19].

Ebselen inhibits ADP-Fe induced lipid peroxidation in hepatocytes, only in the presence of GSH or DTE, and this protection does not appear to be related to a free radical scavenging capacity [12]. The kinetic properties of ebselen account for this inhibition. Indeed this compound that reduces membrane hydroperoxides mimics the reaction of PHGPX, which is a strong inhibitor of lipid peroxidation [20]. In fact the predominant mechanism of initiation of lipid peroxidation in the membranes is the decomposition of lipid hydroperoxides [1]. More precisely this initiation must be referred to as "secondary initiation" because lipid hydroperoxides, that are already peroxidation products, must be previously generated. However, as in the case of PHGPX the prevention of secondary initiations decreases the free radical generation rate and spares the cellular radical scavengers. A cooperation between PHGPX and vit. E in the inhibition of microsomal lipid peroxidation has been observed [21]. It is tempting to speculate that this is also true in the case of ebselen.

The glutathione peroxidase activity of ebselen could support also a role of this compound as a modulator of cyclooxygenases and lipoxygenases. These enzymes are indeed activated by hydroperoxides [8, 9]. In conclusion the peroxidase activity of ebselen supports a possible pharmacological use of this compound as an antiinflammatory and an antileukotriene drug.

REFERENCES

1. S. D. Aust and B. A. Svingen, in *Free Radicals in Biology*, Vol. 5 (Ed. W. A. Pryor), p. 1. Academic Press, New York (1982).
2. F. Haber and J. Weiss, *Proc. R. Soc. Lond.* **147**, 332 (1934).
3. L. A. Witting, in *Free Radicals in Biology*, Vol. 4 (Ed. W. A. Pyror), p. 295. Academic Press, New York (1980).
4. L. Flohé, in *Free Radicals in Biology*, Vol. 5 (Ed. W. A. Pyror), p. 223. Academic Press, New York (1982).
5. L. Flohé and R. Zimmerman, *Biochim. biophys. Acta* **223**, 210 (1970).
6. J. R. Prohaska, S. H. Oh, W. G. Hoekstra and H. E. Ganter, *Biochem. biophys. Res. Commun.* **74**, 64 (1977).
7. F. Ursini, M. Maiorino and C. Gregolin, *Biochim. biophys. Acta* **839**, 62 (1985).
8. P. J. Marshall, R. J. Kulmacz and W. E. M. Lands, *J. biol. Chem.* **262**, 3510 (1987).
9. W. L. Smith and W. E. M. Lands, *J. biol. Chem.* **247**, 1038 (1972).
10. M. J. Parnham, S. Leyck, N. Dereu, J. Winkelman and E. Graf, *Adv. Inflam. Res.* **10**, 397 (1986).
11. A. Mueller, E. Cadenas, P. Graf and H. Sies, *Biochem. Pharmac.* **33**, 3235 (1984).
12. A. Mueller, H. Gabriel and H. Sies, *Biochem. Pharmac.* **34**, 1185 (1985).
13. H. Safayhi, G. Tiegs and A. Wendel, *Biochem. Pharmac.* **34**, 2691 (1985).
14. A. Wendel and G. Tiegs. *Biochem. Pharmac.* **35**, 2115 (1986).

15. L. Flohé, G. Loschen, W. A. Gunzler and E. Eichele, *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 987 (1972).
16. J. Chaudière and Al. L. Tappel, *Archs Biochem. Biophys.* **226**, 448 (1983).
17. S. Daolio, P. Traldi, F. Ursini, M. Maiorino and C. Gregolin, *Biomed. Mass Spectrom.* **10**, 499 (1983).
18. M. Maiorino, A. Roveri, F. Ursini, and C. Gregolin, *J. Free Radicals Biol. Med.* **1**, 203 (1985).
19. H. Fisher and N. Dereu, *Bull. Soc. Chim. Belg.* **96**, 757 (1987).
20. F. Ursini, M. Maiorino, M. Valente, L. Ferri and C. Gregolin, *Biochim. biophys. Acta* **710**, 197 (1982).
21. F. Ursini, M. Maiorino, L. Bonaldo, A. Roveri and C. Gregolin, *Life Chemistry Reports*, (Suppl. **2**), 393 (1984).