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Mechanism of the Reaction of Ebselen with Endogenous Thiols: Dihydrolipoate Is a Better Cofactor than Glutathione in the Peroxidase Activity of Ebselen

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SUMMARY

The therapeutic effect of ebselen has been linked to its peroxidase activity. In the present study, the peroxidase activity of ebselen toward H_2O_2 with the endogenous thiols GSH and dihydrolipoate [$L(SH)_2$] as cofactors was determined. When GSH was used, peroxide removal was described by a ter uni ping pong mechanism with Dalziel coefficients for GSH and H_2O_2 of 0.165 ± 0.011 and 0.081 ± 0.005 mM min, respectively. When $L(SH)_2$ was used, peroxidase activity was independent of the concentration of $L(SH)_2$, in the concentration range studied (5 μM to 2 mM) and peroxide removal was only dependent on the concentration of H_2O_2 and ebselen, with the second-order rate constant being 12.3 ± 0.8 mM^{-1} min^{-1}. To elucidate the difference between GSH and $L(SH)_2$, the molecular mechanism of the peroxidase activity of ebselen was investigated, using UV spectrophotometry, high pressure liquid chromatography, ^{77}Se NMR, and mass spectrometry. GSH was found to react quickly with ebselen to give a selenenyl sulfide, an adduct of GSH to ebselen. Subsequently, the GSH-selenenyl sulfide is converted into the diselenide of ebselen. Finally the diselenide reacts with a peroxide and ebselen is regenerated. The formation by GSH of the diselenide from the GSH-selenenyl sulfide of ebselen is slow and linearly dependent on the concentration of free thiol; however, no net consumption of GSH was observed. Furthermore, it is likely that a selenol is an intermediate in diselenide formation. After reaction between ebselen and $L(SH)_2$ the diselenide of ebselen was immediately detected. The fast formation of the diselenide with $L(SH)_2$ versus the slow formation of the diselenide with GSH accounts for our observation that $L(SH)_2$ is a better cofactor than GSH in the peroxidase activity of ebselen. Our results suggest that the interaction between ebselen and $L(SH)_2$ might be of major importance in the mechanism by which ebselen exerts its therapeutic effect.

In the protection against oxidative stress, GSH-dependent processes play an important role (1). Therefore, one of the strategies in the treatment of various pathologies associated with oxidative stress is to stimulate the GSH-dependent protection. In clinical practice, this might be accomplished by administration of *N*-acetyl-L-cysteine, a precursor of glutathione (2). Another approach is to potentiate the GSH-dependent protection by application of ebselen, a relatively new selenoorganic compound. Ebselen has been shown to be a promising anti-inflammatory drug. However, the mechanism of its anti-inflammatory action is still a matter of debate (3). Ebselen directly inhibits lipoxygenase and cyclooxygenase, it converts leukotriene B_4 into an inactive isomer, and ebselen itself is a potent antioxidant. In addition, ebselen possesses a GSH peroxidase-like activity, using hydrogen peroxide and lipid hydroperoxides as substrate (4, 5). In this study we focus on the peroxidase activity of ebselen.

The selenium-dependent GSH peroxidase reduces the increased "peroxide tone," e.g., in inflammation, by catalyzing the reaction between GSH and hydroperoxides (6, 7, 8). In its peroxidase activity, the enzyme first reacts with a hydroperoxide, a reaction in which the selenol group of the enzyme (*Enz-SeH*) is converted into a selenenic acid (*Enz-SeOH*) and the hydroperoxide into the corresponding alcohol (Fig. 1). Subsequently, the formed selenenic acid reacts with GSH under the formation of a selenenyl sulfide (*Enz-SeSG*). Finally, the selenol form of the enzyme is regenerated by a reaction with a second thiol (Fig. 1) (7).

The selenium-dependent GSH peroxidase selectively uses GSH as cofactor (9). When ebselen is compared with the selenium-dependent GSH peroxidase, ebselen resembles the selenenic acid form of the enzyme. Under *in vivo* conditions, the peroxidase activity of ebselen depends on the reduction of ebselen to its diselenide by thiols (10). Subsequently, the diselenide reacts with a peroxide, with the regeneration of ebselen (10). It has already been reported that synthetic thiols such as dithioerythreitol (11) and *N*-acetyl-L-cysteine (12) can serve as substitutes for GSH in the peroxidase activity of ebselen. Beside GSH, $L(SH)_2$ is an important endogenous SH-containing compound. $L(SH)_2$ is a dithiol with vicinal thiol groups that

ABBREVIATIONS: $L(SH)_2$, dihydrolipoate; DMSO, dimethyl sulfoxide; MSH, mercaptoethanol; HPLC, high pressure liquid chromatography.

can form an intramolecular disulfide in an energetically favorable five-membered ring. $L(SH)_2$ plays a key catalytic role in pyruvate and α-ketoglutarate oxidase. Like GSH, $L(SH)_2$ provides protection against free radical-mediated injury. Recently, an interplay between $L(SH)_2$ and GSH in reducing the peroxide tone by protecting against lipid peroxidation has been demonstrated (13, 14). The aim of this investigation is to determine whether $L(SH)_2$ is also accepted by ebselen as cofactor and, furthermore, to study the mechanism of the peroxidase activity of ebselen.

Materials and Methods

GSH was obtained from Sigma (St. Louis, MO). Ebselen [2-phenyl-1,2-benzisoselenazol-3-(2*H*)-one] and ebselen derivatives were gifts of Rhône-Poulenc Nattermann (Cologne, FRG). Racemates of dihydrolipoic acid [(±)-6,8-dimercaptooctanoic acid] and lipoic acid [(±)-1,2-dithiolane-3-pentanoic acid] were gifts of Asta Pharma AG (Frankfurt, FRG). All other chemicals were of analytical grade purity.

All incubations were performed at 37° in a 10 mM potassium phosphate buffer, pH 7.4, unless otherwise stated. Ebselen was dissolved in DMSO. Maximal concentration of DMSO was 1%.

Hydrogen peroxide was determined with the iron-thiocyanate method according to the method of Hildebrandt and Roots (15). This method is based on the oxidation of Fe^{2+} to Fe^{3+} by H_2O_2. The formed Fe^{3+}, complexed to thiocyanate, is quantified spectrophotometrically at 480 nm. Free thiol groups were assessed according to the method of Ellman (16).

The HPLC analysis of ebselen, GSH-selenenyl sulfide, and diselenide of ebselen was basically according to the methods of Müller *et al.* (17) and Terlinden *et al.* (18). Samples of 20 μl were injected onto a reverse phase column (Nucleosil C18; Chrompack, Middelburg, The Netherlands) and the products were monitored by UV absorption at 313 nm. The mobile phases consisted of mixtures of acetonitrile (A) and 0.1% H_3PO_4 (B) at the flow rate of 0.6 ml/min. A mixture of 50% A and 50% B was used to elute ebselen (retention time, 2.90 min); a mixture of 30% A and 70% B was used for the GSH-selenenyl sulfide (retention time, 3.12 min); a mixture of 50% A and 50% B was used for the MSH-selenenyl sulfide (retention time, 3.30 min); and a mixture of 70% A and 30% B was used for the diselenide (retention time, 3.75 min).

All ^{77}Se NMR spectra were recorded on a Bruker MSL 400, in a tube of 7 mm outer diameter, in dimethyl formamide at room temperature. Chemical shifts are reported relative to dimethylselenide. Ebselen served as reference, with a chemical shift of 959 ppm (10).

Mass spectrometric analysis was carried out on a Finnigan MAT 90 mass spectrometer. Samples were directly introduced into the mass spectrometer. Direct chemical ionization was performed, using isobutane as the reagent gas and an ion source temperature of 50°, at a pressure of 10^{-4} Torr.

In principle, all results are expressed as mean ± standard deviation (three to five experiments). In most experiments, the standard deviation was smaller than 5% and was omitted for the sake of clarity.

Results

As shown in Fig. 2, $L(SH)_2$ did not react spontaneously with hydrogen peroxide at a measurable rate. However, in combination with ebselen, $L(SH)_2$ degraded hydrogen peroxide in the incubation medium very quickly. PZ 25 (RP 62373), an analog of ebselen in which selenium has been replaced by sulfur, proved to have no catalytic activity in the reaction between $L(SH)_2$ and hydrogen peroxide (data not shown). It is also shown in Fig. 2 that GSH, in contrast to $L(SH)_2$, reacted spontaneously with hydrogen peroxide; however, GSH was found to be less potent, compared with $L(SH)_2$, in hydrogen peroxide removal when ebselen was present. When $L(SH)_2$ was used, the rate of hydrogen peroxide removal could be described by a second-order reaction (rate constant, 12.3 ± 0.8 mM^{-1} min^{-1}), in which the rate of hydrogen peroxide removal was linearly dependent on both the concentration of hydrogen peroxide and the concentration of ebselen but independent of the concentration of $L(SH)_2$, in the concentration range investigated (5 μM to 2 mM) (Fig. 3). Lipoate, the oxidized form of $L(SH)_2$, was not effective as cofactor in mediating the peroxidase activity of ebselen (data not shown).

To elucidate the cause of the difference between GSH and $L(SH)_2$ in the peroxidase activity of ebselen, the reactivity of ebselen towards both thiols was determined. In the UV spectrum of ebselen, the absorption maximum at 324 nm is due to the isoselenazol ring (4). As shown in Fig. 4A, addition of 12.5, 25, 37.5, or 50 μM GSH to 50 μM ebselen resulted in a concentration-dependent reduction of the absorption at 324 nm, indicating that GSH opens the isoselenazol ring (cf. Ref. 4). The isobestic point at 318 nm indicates that a relatively stable product or a mixture of stable products of constant composition was formed. When more GSH than ebselen was added to the reaction mixture and the UV spectrum was immediately recorded, the spectrum was identical to the spectrum obtained after the addition of an equimolar amount of GSH and ebselen. It was noted that the spectrum recorded several minutes after the addition of an excess of GSH differed from the spectrum obtained immediately after the addition of the excess of GSH (Fig. 4A, *curve 6*). This indicates that, after the addition of an

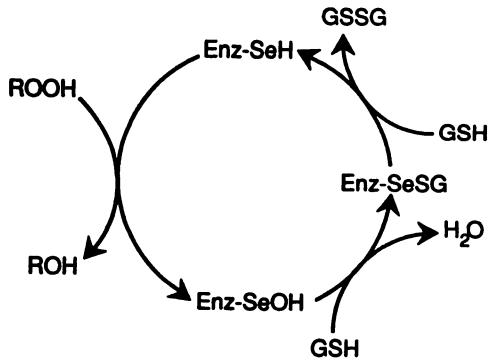

Fig. 1. Reaction scheme of the endogenous selenium-dependent GSH-peroxidase. Hydroperoxides (*ROOH*) are reduced to the corresponding alcohols at the expense of GSH by a ter uni ping pong mechanism.

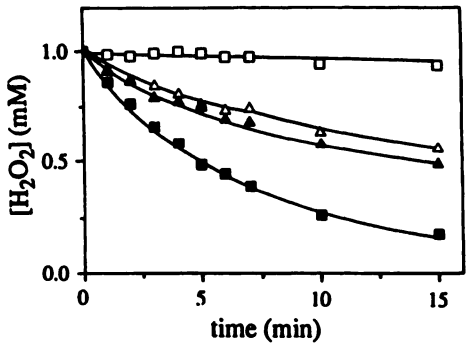

Fig. 2. Reaction of 0.5 mM $L(SH)_2$ (*squares*) or 2 mM GSH (*triangles*) with 1 mM hydrogen peroxide. In the experiments depicted by the *closed symbols*, 10 μM ebselen was added. The reaction was started by the addition of hydrogen peroxide.

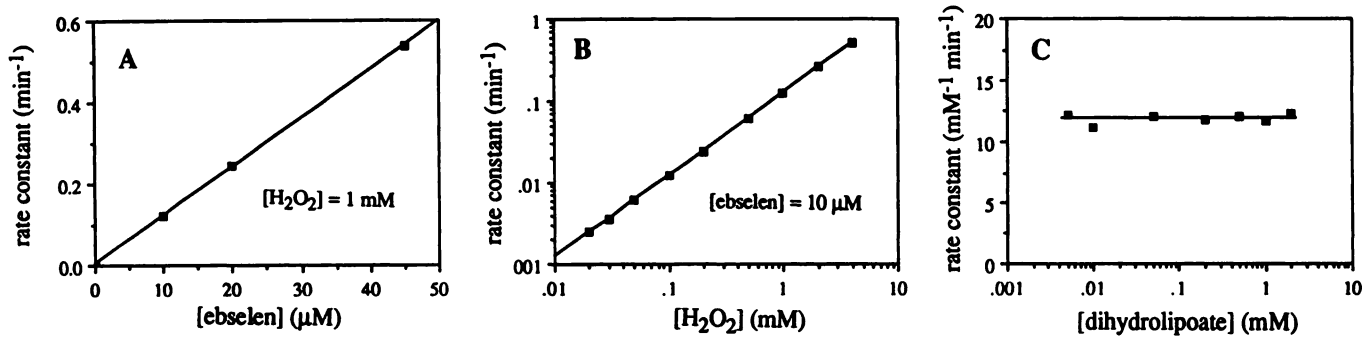

Fig. 3. Peroxidase activity of ebselen with $L(SH)_2$ as cofactor. A, Dependence of hydrogen peroxide removal on the concentration of ebselen; the pseudo-first-order rate constant with an initial concentration of hydrogen peroxide of 1 mM is shown. B, Dependence on the concentration of hydrogen peroxide; the pseudo-first-order rate constant with a concentration of ebselen of 10 μM is shown. C, Dependence on the concentration of $L(SH)_2$; various concentrations of hydrogen peroxide and ebselen were used and the second-order rate constant (k) was determined using the formula: $d[H_2O_2]/dt = -k[H_2O_2]$ [ebselen]. The second-order rate constant (k) appeared to be 12.3 ± 0.8 mM^{-1} min^{-1}.

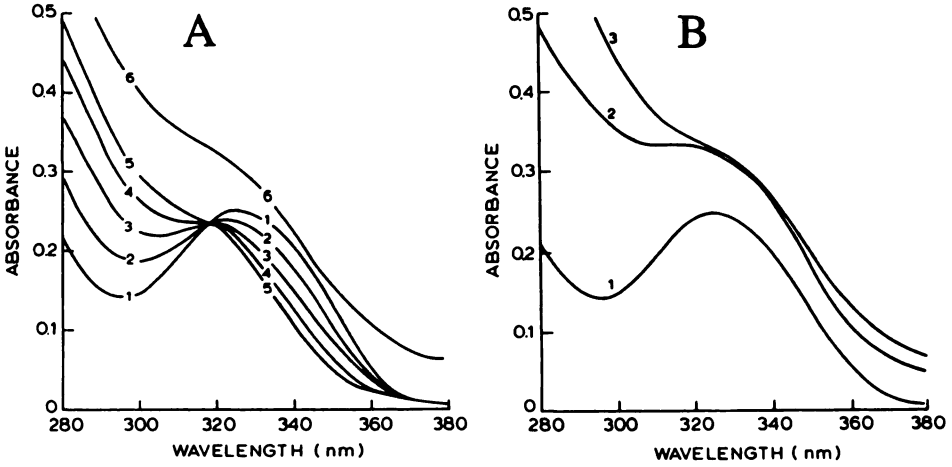

Fig. 4. Change in the UV absorption spectrum of 50 μM ebselen by the addition of GSH (A) or $L(SH)_2$ (B). The concentrations of GSH (A) were 0 (*1*), 12.5 (*2*), 25 (*3*), 37.5 (*4*), 50 (*5*), or 60 μM (*6*). The concentrations of $L(SH)_2$ (B) were 0 (*1*), 12.5 (*2*), or 25 μM (*3*). All spectra were recorded immediately after the addition of the thiol, except for *spectrum 6* in A, which was recorded 10 min after the addition of 60 μM GSH.

excess of GSH to ebselen, a reaction product is being formed at a relatively low rate. When $L(SH)_2$ instead of GSH was used, even at relatively low concentrations of $L(SH)_2$ the spectrum that was observed with an excess of GSH only after several minutes appeared immediately (Fig. 4B).

In order to find out which products correspond to the observed UV absorptions, the reaction mixtures of ebselen and either GSH or $L(SH)_2$ were analyzed by HPLC. A sample of the reaction mixtures taken immediately after the addition of the thiol was injected onto the HPLC column. In the case of GSH it was found that the GSH-selenenyl sulfide of ebselen [*S*-(2-phenyl carbamoyl benzeneselenenyl)-glutathione] was formed (Fig. 5). Only with relatively high concentrations of GSH was some diselenide of ebselen [2,2-diselenobis-(*N*-phenyl-benzamid)] detected. The reaction between GSH and ebselen was fast and complete, with 1 mol of GSH consuming 1 mol of ebselen and the major product being the GSH-selenenyl sulfide of ebselen.

When the ebselen-GSH mixture was incubated for longer periods, the GSH-selenenyl sulfide gradually converted into the diselenide of ebselen. The rate of diselenide production was strongly dependent on the GSH concentration (Fig. 6). When ebselen was added in excess to GSH, no free GSH was detected (Fig. 5) and the diselenide was formed slowly (Fig. 6). When GSH was added in excess to ebselen, diselenide formation was much faster. No extra thiol was consumed in the diselenide formation (data not shown). Diselenide formation followed second-order rate kinetics with respect to the concentration of

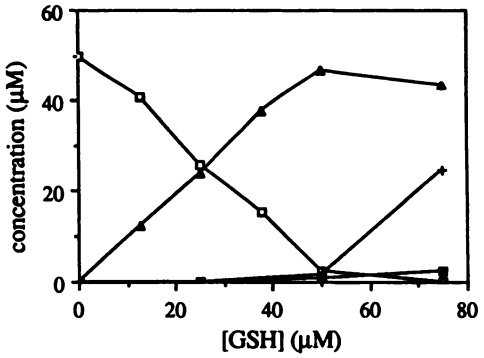

Fig. 5. Formation of the GSH-selenenyl sulfide of ebselen (▲) and the diselenide (■) from 50 μM ebselen (□) and a varying concentration of GSH; +, concentration of free sulfhydryl groups. The reaction mixtures were analyzed 15 sec after GSH was added, using HPLC.

the GSH-selenenyl sulfide of ebselen and the concentration of free GSH. The rate constant of diselenide formation was found to be 6.1 ± 0.4 mM^{-1} min^{-1} (Fig. 6). Addition of GSSG did not decrease the GSH-mediated diselenide formation from ebselen (data not shown).

Variation of the pH of the incubation medium showed that the rate of diselenide formation decreased when the pH declined. Using a pK_a of the thiol of GSH of 8.6 (19) for calculation, it was found that the second-order rate constant correlated with the fraction of deprotonated GSH (not shown). This

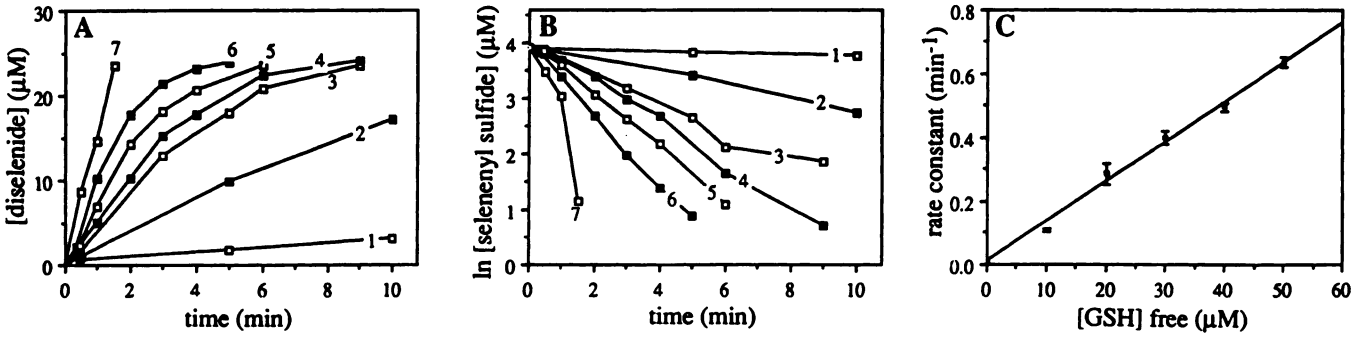

Fig. 6. Time course of the diselenide (A) and the selenenyl sulfide (B) concentration after the addition of various amounts of GSH to 50 μM ebselen. The concentrations of GSH added were 50 (*1*), 60 (*2*), 70 (*3*), 80 (*4*), 90 (*5*), 100 (*6*), and 150 μM (*7*). In C, the dependence of the rate of selenenyl sulfide consumption on the concentration of free GSH is shown.

indicates that the deprotonated form of GSH is the active species.

When $L(SH)_2$ was used in the reaction with ebselen instead of GSH, the diselenide of ebselen was immediately detected using HPLC. No selenenyl sulfides or other intermediates could be detected. The reaction between the dithiol $L(SH)_2$ and ebselen to form the diselenide was fast and complete, with 1 mol of $L(SH)_2$ consuming 2 mol of ebselen (Fig. 7).

In order to further identify the products of the reaction between ebselen and $L(SH)_2$, we studied the reaction mixtures with ^{77}Se NMR. The natural abundance of ^{77}Se is 7.6% of the total amount of selenium, and ^{77}Se can be used to monitor reactions of ebselen by NMR. However, due to the low sensitivity of ^{77}Se NMR, relatively high concentrations are needed. Because ebselen is only poorly soluable in aqueous solutions, the NMR experiments were conducted in dimethyl formamide, similar to experiments by Fischer and Dereu (10). When $L(SH)_2$ was added to ebselen, only the diselenide of ebselen could be detected by ^{77}Se NMR. No other products or intermediates were observed (not shown). Comparable to the HPLC data, this technique also showed that 1 mol of $L(SH)_2$ consumed 2 mol of ebselen (not shown).

The formation of the diselenide in the reaction between ebselen and $L(SH)_2$ was confirmed using direct chemical ionization-mass spectrometry. To 1 ml of a solution of 18 μmol of ebselen in DMSO, 2 ml of a solution of 9 μmol of $L(SH)_2$ in 10 mM potassium phosphate buffer, pH 7.4, was added. A sample of this reaction mixture was immediately analyzed, and it was found that the positive chemical ionization-mass spectrum of the formed product (Fig. 8) had the same characteristics as the mass spectrum obtained from a reference sample of the diselenide (not shown). The cluster of ions at *m/z* 545–557 corresponds to the calculated pattern of isotopes of a compound with the same chemical composition as the protonated diselenide ($C_{26}H_{21}N_2O_2Se_2$). The base peak of the ion at *m/z* 207, which is derived from lipoic acid, also indicated that $L(SH)_2$ is oxidized in a reaction with ebselen.

To answer the question of why $L(SH)_2$ is a better cofactor than GSH in the reaction with ebselen, two types of experiments were conducted. In the first experiment, the effect of GSH addition on the removal of hydrogen peroxide by the combination of $L(SH)_2$ and ebselen was determined. To the incubation medium, first GSH (1 mM) was added, then ebselen (10 μM), and subsequently $L(SH)_2$ (0.5 mM). The reaction was started by the addition of hydrogen peroxide (0.5 mM). It was found that GSH did not reduce the $L(SH)_2$ supported peroxidase activity of ebselen (data not shown).

In the second experiment, 50 μM GSH was added to 50 μM ebselen, which resulted in a rapid formation of the GSH-selenenyl sulfide of ebselen (Fig. 5). When $L(SH)_2$ was also added, 15 sec after the addition of GSH, it was found that the diselenide of ebselen was immediately formed (Fig. 9). Apparently, GSH is less effective than $L(SH)_2$ in the generation of

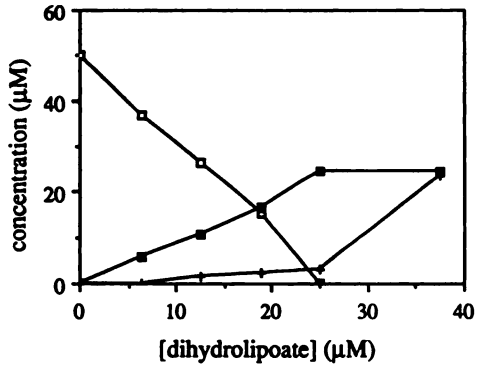

Fig. 7. Formation of the diselenide (■) from 50 μM ebselen (□) and a varying concentration of $L(SH)_2$. +, concentration of free sulfhydryl groups. The reaction mixtures were analyzed 15 sec after $L(SH)_2$ was added using HPLC.

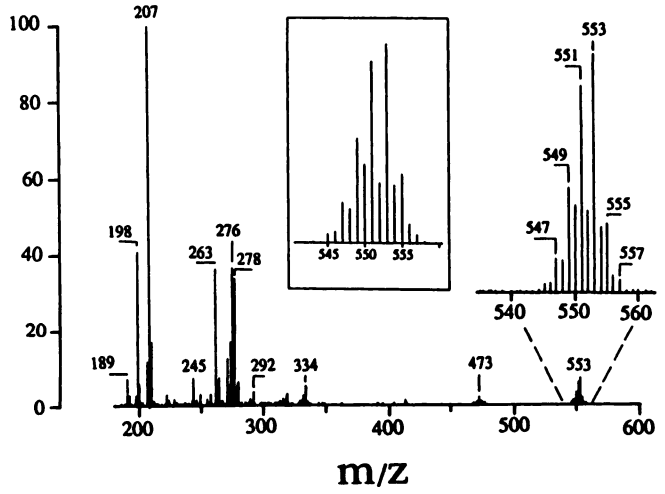

Fig. 8. Mass spectrum of the products of the reaction between ebselen and $L(SH)_2$. To 18 μmol of ebselen in 1 ml of DMSO, 2 ml of an aqueous solution of 9 μmol of $L(SH)_2$ were added. After the addition of $L(SH)_2$ a sample of the reaction mixture was directly introduced into the mass spectrometer. In the *inset*, the calculated mass spectrum of a compound with the chemical composition of the protonated diselenide is depicted.

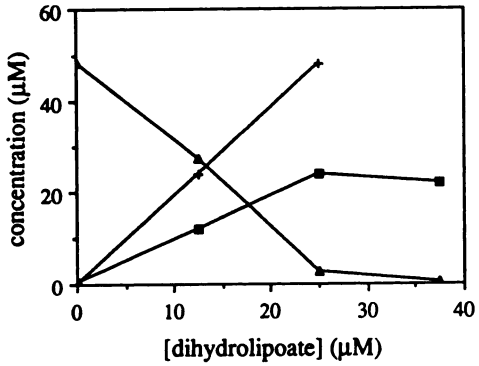

Fig. 9. Formation of the diselenide (■) from the GSH-selenenyl sulfide (▲) of ebselen and a varying concentration of $L(SH)_2$. The GSH-selenenyl sulfide is generated by the addition of 50 μM GSH to 50 μM ebselen. After 15 sec, $L(SH)_2$ in the given concentrations was added. Subsequently, after another 15 sec, the reaction mixture was analyzed using HPLC; +, concentration of free sulfhydryl groups detected.

the diselenide from the GSH-selenenyl sulfide of ebselen (Figs. 5 and 6).

In order to elucidate the mechanism of the reaction between $L(SH)_2$ and the GSH-selenenyl sulfide of ebselen, which immediately forms the diselenide, the interaction of ebselen with the synthetic thiol MSH was investigated. Similar to GSH, MSH reacted rapidly with ebselen, with 1 mol of MSH consuming 1 mol of ebselen and the major product being the MSH-selenenyl sulfide of ebselen. When various concentrations of MSH were used, the concentration of ebselen was kept constant, and the reaction mixture was analyzed immediately after the addition of MSH by HPLC, the results were identical to those obtained with GSH, as depicted in Fig. 5, albeit that the MSH-selenenyl sulfide instead of the GSH-selenenyl sulfide was formed. Diselenide formation followed second-order rate kinetics with respect to the concentration of the MSH-selenenyl sulfide of ebselen and the concentration of free MSH (rate constant, 2.2 ± 0.1 mM^{-1} min^{-1}).

When 50 μM GSH was added to 50 μM ebselen, all ebselen was immediately converted into the GSH-selenenyl sulfide. As described above, diselenide formation with equimolar amounts of GSH and ebselen was slow. Addition of 50 μM MSH to this incubation mixture, 15 sec after the addition of GSH, yielded the MSH-selenenyl sulfide of ebselen and also enhanced diselenide formation (Fig. 10).

Comparable results were obtained when MSH (50 μM) was added to ebselen (50 μM) 15 sec before GSH (50 μM). Approximately half of the MSH-selenenyl sulfide was converted into the GSH-selenenyl sulfide and diselenide formation was faster, compared with the incubation system without MSH. Also, when GSH (50 μM) and MSH (50 μM) were mixed and the reaction was started by the addition of ebselen (50 μM), the compounds detected in the incubation mixture were the diselenide and the selenenyl sulfides of ebselen with both MSH and GSH. When a combination of MSH and GSH was used, the concentrations of the selenenyl sulfide of ebselen with either GSH or MSH were independent of the way the reaction was started. Diselenide formation was fastest with 100 μM GSH and slowest with 100 μM MSH. An intermediate rate of diseleneide formation was observed when both 50 μM GSH and 50 μM MSH were added to the reaction mixture (Fig. 10).

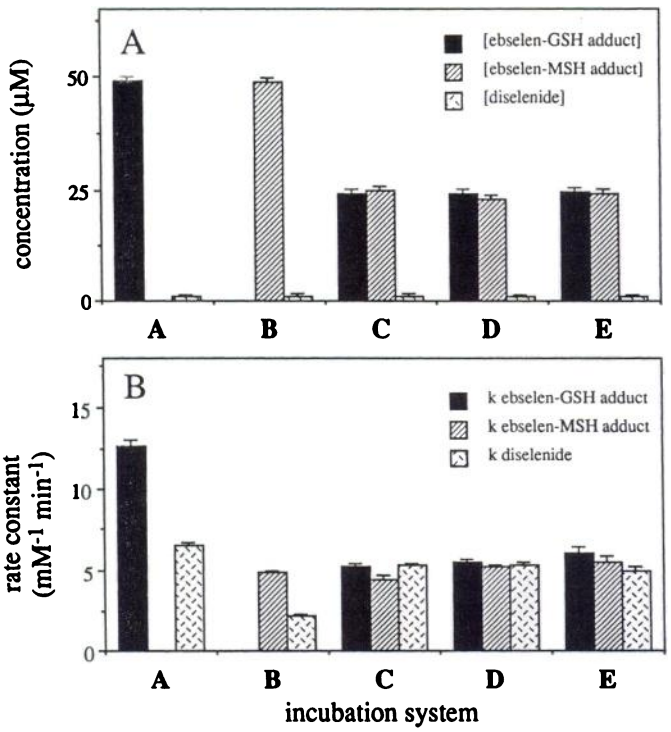

Fig. 10. Reaction of the combination of GSH and MSH with ebselen. In *panel* A, the concentration of GSH-selenenyl sulfide of ebselen ([*ebselen-GSH adduct*]), the concentration MSH-selenenyl sulfide of ebselen ([*ebselen-MSH adduct*]), and the concentration of diselenide ([*diselenide*]) are shown. In all incubation systems, except E, first 50 μM ebselen was added. In incubation system A, 100 μM GSH was added; in incubation system B, 100 μM MSH was added; in incubation system C, 50 μM GSH and, 15 sec thereafter, 50 μM MSH were added; in incubation system D, 50 μM MSH and, 15 sec thereafter, 50 μM GSH were added. In incubation system E, 50 μM GSH and 50 μM MSH were added and 15 sec thereafter, 50 μM ebselen was added. The reaction mixtures were analyzed with HPLC 15 sec after the last addition. In *panel* B, the rate of disappearance of the GSH-selenenyl sulfide of ebselen (*k ebselen-GSH adduct*), the rate of disappearance of the MSH-selenenyl sulfide of ebselen (*k ebselen-MSH adduct*), and the rate of diselenide formation (*k diselenide*) for the same incubation systems as in *panel* A are depicted.

Discussion

Ebselen is a selenium-containing heterocyclic compound, which displays anti-inflammatory activity (6, 8). Like the endogenous selenium-dependent GSH peroxidase, ebselen catalyzes the reaction between GSH and peroxides, and this catalytic activity is probably linked to the therapeutic effect of ebselen (4, 5). In general, GSH is regarded as the cofactor for the perxoidase activity of ebselen. Remarkably, in the present study we found that the endogenously occurring thiol $L(SH)_2$ is a better cofactor than GSH in the peroxidase activity of ebselen (Fig. 2). In contrast to GSH, $L(SH)_2$ did not react spontaneously with hydrogen peroxide. However, in the ebselen-catalyzed reduction of hydrogen peroxide, $L(SH)_2$ proved to be far more effective than GSH (Fig. 2). Our results indicate that, with either GSH or $L(SH)_2$, ebselen is reduced to its diselenide, but the rate of this reaction differs considerably for the two thiols.

In a recently proposed scheme by Fischer and Dereu (10), the reaction between ebselen and thiols in the first instance yields a selenenyl sulfide. Subsequently, the selenenyl sulfide slowly undergoes a cleavage reaction to the diselenide and disulfide. This reaction was supposed to be an equilibrium, with

the equilibrium constant depending on the nature of the thiol and the pH of the medium and the reaction being independent of any selenol intermediate (10).

In accordance with the mechanism proposed by Fischer and Dereu (10), we found that, in the reaction between GSH and ebselen, selenenyl sulfide formation was fast, whereas the diselenide formation was slow (Fig. 5). However, we also found that diselenide formation proceeds via a second-order reaction, because the rate of diselenide formation was linearly dependent on the concentration of free GSH and the concentration of selenenyl sulfide (Fig. 6). The second-order rate kinetics do not fit the equilibrium reaction between the selenenyl sulfide and the diselenide presented by Fischer and Dereu (10). Additionally, we did not observe a diminished diselenide formation with GSH when GSSG was added, which also argues against the equilibrium proposed by Fischer and Dereu (10).

Based on the present data, we propose a modified reaction scheme for the GSH peroxidase activity of ebselen, as depicted in Fig. 11. In this mechanism, the nucleophile GSH attacks the selenium atom of ebselen and substitutes the amide nitrogen with the formation of a selenenyl sulfide. Subsequently, the sulfur atom of the selenenyl sulfide is attacked by a second GSH molecule with the formation of an as yet unidentified selenol and GSSG. Of the two substitution reactions by GSH, the second is relatively slow (second order rate constant, 6.1 ± 0.1 mM^{-1} min^{-1}) and can be rate determining in the peroxidase activity of ebselen. The second-order kinetics of this reaction indicate that it involves a S_N2-type substitution reaction in which the selenol of ebselen is the leaving group. Because no selenol could be detected, it is likely that the selenol reacts rapidly with a second selenenyl sulfide. By this nucleophilic attack of the selenol at the selenium atom of the selenenyl sulfide, GSH is released from the selenenyl sulfide and a diselenide of ebselen is formed.

In agreement with Fischer and Dereu (10), in the conversion of the GSH-selenenyl sulfide to the diselenide of ebselen, the net reaction is that two selenenyl sulfides form one diselenide and GSSG. In contrast to the scheme presented by Fischer and Dereu (10), the diselenide is not generated from the selenenyl sulfide by the equilibrium reaction as proposed by them, but by two S_N2-type nucleophilic substitutions, firstly by GSH at the sulfur atom of the selenenyl sulfide, with the formation of a selenol intermediate, and subsequently by this selenol at the selenium atom of a second selenenyl sulfide, in which the diselenide of ebselen and GSSG are formed. GSH is, thus, a catalyst in the formation of the diselenide from the selenenyl sulfide. In both nucleophilic substitution reactions, the deprotonated thiol or selenol is most likely the reactive species (19). Finally, the diselenide reacts with hydrogen peroxide and ebselen is regenerated. A selenenic acid anhydride has been reported to be an intermediate in this reaction (10). Recently, Maiorino *et al.* (20) were able to trap a selenol intermediate with the electrophile iodoacetamide in the reaction between GSH and ebselen, which further supports the modified reaction scheme we present (Fig. 11).

When $L(SH)_2$ was used instead of GSH in the reaction with ebselen, a rapid diselenide formation was found (Fig. 7). That indeed the diselenide was formed was confirmed using ^{77}Se NMR (not shown) and mass spectrometry (Fig. 8). Previously, Müller *et al.* (17) generated a mass spectrum of the diselenide using fast atom bombardment. We applied direct chemical ionization, which appeared to be a more convenient technique for the diselenide. In the direct chemical ionization mass spectrum, the cluster of ions with m/z 545–557 perfectly matched the theoretical isotope distribution of a compound with the chemical composition of the protonated diselenide (Fig. 8).

Based on these results and in analogy with the reaction

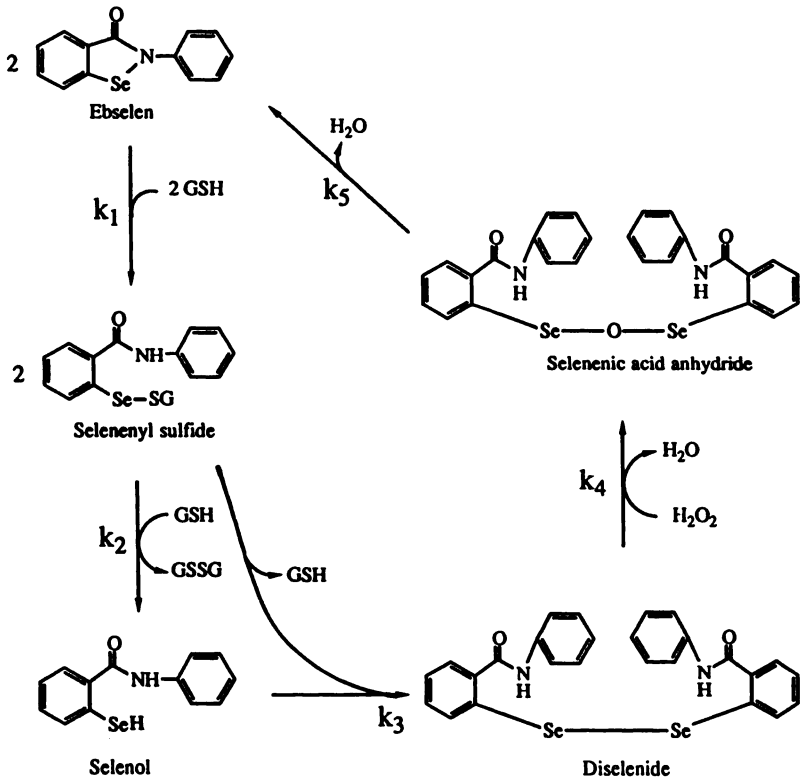

Fig. 11. Proposed reaction scheme for the GSH-mediated peroxidase activity of ebselen. The reaction between GSH and the GSH-selenenyl sulfide or the reaction between H_2O_2 and the diselenide is rate determining in the GSH-supported peroxidase activity of ebselen. In contrast to the scheme presented by Fischer and Dereu (10), a selenol intermediate is suggested to be involved.

between ebselen and GSH, we propose a reaction scheme for the $L(SH)_2$-supported peroxidase activity of ebselen (Fig. 12) in which ebselen first reacts with the dithiol $L(SH)_2$ to yield a selenenyl sulfide intermediate. In contrast to the reaction with GSH, the $L(SH)_2$-selenenyl sulfide rapidly forms a selenol (Fig. 7), probably because a free sulfhydryl group is available in the $L(SH)_2$-selenenyl sulfide that functions as an intramolecular nucleophile. Subsequently, the formed selenol reacts with ebselen or with the $L(SH)_2$-selenenyl sulfide with the formation of the diselenide of ebselen. Similar to the reaction between ebselen and GSH, the reaction between ebselen and $L(SH)_2$ is most likely a nucleophilic substitution by one of the sulfhydryl groups of $L(SH)_2$ at the selenium atom of ebselen. In principle, either one of the sulfhydryl groups of $L(SH)_2$ may attack the selenium atom. As described above, the deprotonated thiol is most likely the nucleophile involved in this type of substitution reaction. In general, it is known that both the extent of ionization of a thiol and the intrinsic nucleophilicity of the corresponding thiolate anion determine the overall reactivity of thiols in this type of nucleophilic reactions. It has been suggested that the lower the pK_a of a thiol the lower the nucleophilicity of the thiolate, but the higher the relative concentration of thiolate (19, 21, 22). When the effect of the pH on the reactivity of thiols in S_N2 reactions was studied, it was found that, of the two opposite effects of the pK_a on the reactivity, the effect of the pK_a on the fraction of thiol that is deprotonated contributes more, if $pH < pK_a$ (21, 22). This means that the thiol in $L(SH)_2$ with the lower pK_a would be the better nucleophile in our experimental set-up.

Unpublished results indicated that the pK_a values of the two thiol groups in $L(SH)_2$ are virtually identical, indicating that the ionized fraction of both thiols and the nucleophilicity of the respective thiolate anions do not differ very much. We also found that the two sulfhydryl groups influence each other, because deprotonation of one thiol increased the pK_a of the other sulfhydryl group. A comparable change in the pK_a value of other thiols is known. Upon protonation of its amine, the pK_a of the thiol in cysteine decreased from 10.6 to 8.6 (23). It should also be noted that steric hindrance might reduce the nucleophilic reactivity of the secondary thiol in $L(SH)_2$. Because the two different $L(SH)_2$-selenenyl sulfides of ebselen were too reactive to be isolated and analyzed, we were not able to determine the relative contribution of each thiol in the reaction with ebselen. Moreover, intramolecular conversion of one selenenyl sulfide into the other [comparable to the intramolecular conversion of 8-*S*-acetyldihydrolipoamide into 6-*S*-acetyldihydrolipoamide and vice versa (24)] might hamper such an attempt. As a result of substitution to the selenium atom of ebselen by one of the thiol groups of $L(SH)_2$, the pK_a of the other free thiol in the $L(SH)_2$-selenenyl sulfide of ebselen will probably drop. If the presumed relation between pK_a and nucleophilicity holds, the free thiol group would be rendered a stronger nucleophile, which would enhance the nucleophilicity of this thiol and hence the rate of selenol and subsequent diselenide formation.

The large difference in the rate of diselenide formation in the reaction between ebselen and GSH or $L(SH)_2$ accounts for our observation that $L(SH)_2$ is a better cofactor than GSH in the peroxidase activity of ebselen. When $L(SH)_2$ is used instead of GSH, selenol formation is no longer rate limiting. With $L(SH)_2$ as cofactor, hydrogen peroxide removal is governed by the second-order reaction between the diselenide and hydrogen

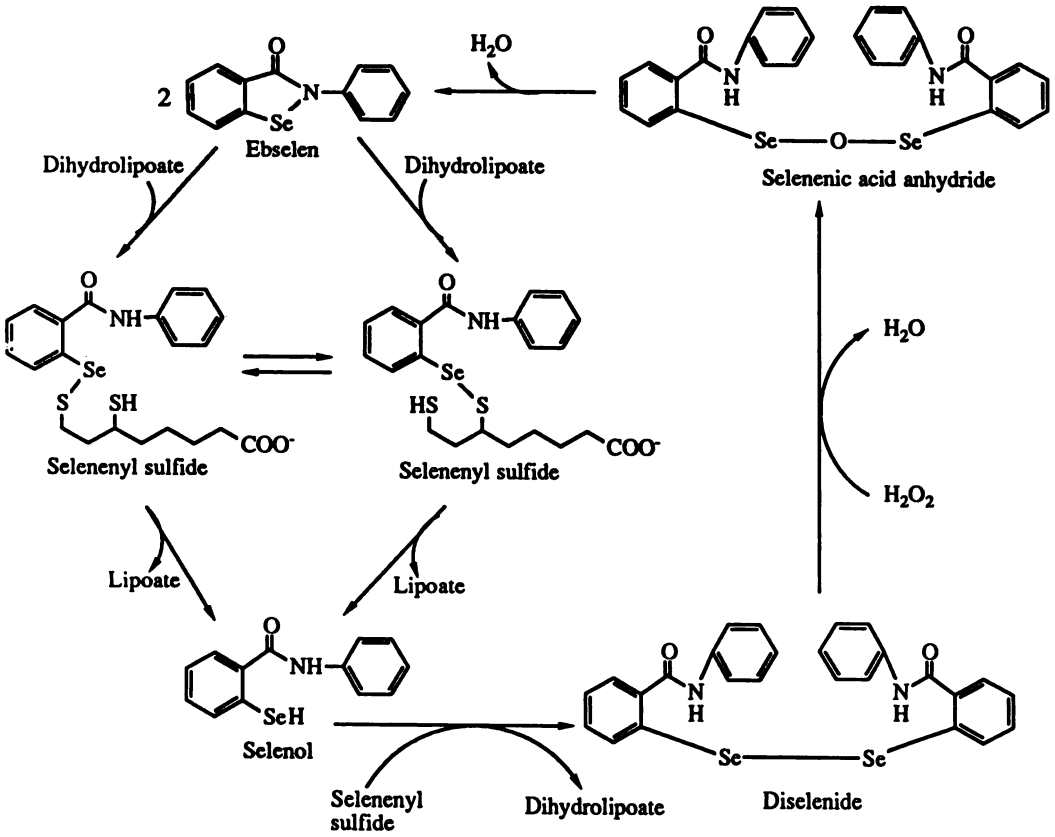

Fig. 12. Proposed reaction scheme for the $L(SH)_2$-mediated peroxidase activity of ebselen. The reaction between H_2O_2 and the diselenide is rate determining in the $L(SH)_2$-supported peroxidase activity of ebselen. In the formation of the diselenide from the selenol, only the reaction of the selenol with the $L(SH)_2$-selenenyl sulfide is depicted. Alternatively, the diselenide might also be formed in a reaction of the selenol with ebselen, a reaction omitted for the sake of clarity.

peroxide (Fig. 3). In this case the rate of peroxide removal (V) can be described by the following equation:

$$V = \frac{-d[H_2O_2]}{dt} = 12.3 \times 10^{-3}[H_2O_2][Ebs](\text{M min}^{-1})$$

In this equation, [Ebs] is the concentration of ebselen added to the incubation mixture.

According to our reaction scheme for the GSH-mediated peroxidase activity of ebselen (Fig. 11), the rate of peroxide removal with GSH as cofactor can be described by the following equation (see Appendix):

$$V = \left\{\frac{2}{k_1[GSH]} + \frac{1}{k_2[GSH]} + \frac{2}{k_4[H_2O_2]} + \frac{2}{k_5}\right\}^{-1}\left\{[Ebs] - \frac{k_2[GSH]}{k_3}\right\}$$

Because, in diselenide formation, the reaction between GSH and the selenenyl sulfide is rate limiting (i.e., $k_1 \gg 2k_2$ and $k_3 \gg k_2$ [GSH]) and because the reaction between the diselenide and hydrogen peroxide can be described by a second-order reaction (i.e., $k_5 \gg k_4$ [H_2O_2]), the equation can be simplified to:

$$V = \left\{\frac{1}{k_2[GSH]} + \frac{2}{k_4[H_2O_2]}\right\}^{-1}[Ebs]$$

In this equation, k_2 is half of the overall rate of selenenyl sulfide consumption as calculated in Fig. 6, thus, k_2 is 6.1 ± 0.4 mM^{-1} min^{-1}, and k_4 is twice the second-order rate constant that describes peroxidase activity with ebselen and $L(SH)_2$, thus, k_4 is 24.6 ± 1.6 mM^{-1} min^{-1}. Interestingly, this simplified equation is comparable to the Dalziel equation that describes peroxide removal by the endogenous selenium-dependent GSH peroxidase (Fig. 1) (25). The reaction between ebselen and either substrate, hydroperoxide or GSH, can be rate limiting. The K_m and V_{max} for these substrates are not constant; the apparent kinetic coefficients of one substrate are a function of the concentration of the other substrate. The Dalziel constants of ebselen for GSH ($=1/k_2$) and hydrogen peroxide ($=2/k_4$) found in this study are respectively 0.165 ± 0.011 mM min and 0.081 ± 0.005 mM min at pH 7.4, 37°. Recently, Maiorino *et al.* (20) also reported that the ebselen-catalyzed reaction between GSH and peroxides can be described by a ter uni ping-pong mechanism. They stated that the Dalziel coefficients of ebselen with hydrogen peroxide as substrate were 0.66 mM min for GSH and 0.015 mM min for hydrogen peroxide. They also determined the Dalziel coefficients of ebselen with other hydroperoxides than hydrogen peroxide as substrate. Surprisingly, they found that the Dalziel coefficient for GSH depended on the hydroperoxide used because the coefficient varied from 1.4 to 0.073 mM min (20). In principle, this Dalziel coefficient describes the reaction between GSH and ebselen, a reaction that is independent of the hydroperoxide used. For comparison, the Dalziel coefficient of the selenium-dependent GSH peroxidase for GSH is indeed independent of the hydroperoxide used (25). The Dalziel coefficient of ebselen for GSH we found is within the range Maiorino *et al.* (20) reported.

Our data clearly show that the $L(SH)_2$-mediated formation of the diselenide of ebselen is much faster (Fig. 7), when compared with the corresponding GSH-supported reaction (Figs. 5 and 6). At first sight, the most likely explanation for this difference is the availability of a second intramolecular nucleophilic sulfhydryl group in the $L(SH)_2$-selenenyl sulfide of ebselen, in the vicinity of the electrophilic sulfur atom attached to selenium in the selenenyl sulfide. Alternatively, $L(SH)_2$ might be a better cofactor than GSH in the peroxidase activity of ebselen simply because $L(SH)_2$ is a better reducing agent. The redox potential of the couple $L(SH)_2$-lipoate is −0.32 V, versus −0.24 V for the couple GSH-GSSG (19). However, it should be noted that, just because a compound is a better reducing agent, this does not imply that redox reactions with this reducing agent go faster, compared with reactions with a less potent reducing agent. For example, in the spontaneous reduction of hydrogen peroxide, the activation energy of the redox reaction with $L(SH)_2$ proved to be higher than that with GSH (Fig. 2).

In vivo both GSH and $L(SH)_2$ are present. GSH might reduce the $L(SH)_2$-mediated activity of ebselen because ebselen might be entrapped as GSH-selenenyl sulfide. Therefore, the effect of GSH on the $L(SH)_2$-mediated peroxide removal by ebselen was also determined. The contribution of each thiol might not be determined by the overall reaction rate of diselenide formation out of ebselen by each thiol but by the reaction rate of ebselen with each of the thiols, a reaction that is fast for both thiols. The pK_a of the sulfhydryl group of GSH (8.6–8.9) (19) is higher than the pK_a of both the sulfhydryl groups of $L(SH)_2$.[1] This implies that at pH 7.4 more $L(SH)_2$ than GSH is deprotonated and, thus, it would be expected that the reaction of ebselen with $L(SH)_2$ proceeds faster than the reaction of ebselen with GSH. However, at a pH of 7.4 the chemical reactivity of GSH was found to be higher, compared with that of $L(SH)_2$ (data not shown), indicating that ebselen would react faster with GSH than with $L(SH)_2$. Moreover, in our experiment GSH was added before $L(SH)_2$, so the formation of the GSH-selenenyl sulfide was favored, and also during the actual peroxidase activity the GSH-selenenyl sulfide might be formed. Nevertheless, it was found that GSH did not reduce the $L(SH)_2$-supported peroxidase activity of ebselen.

The rate of formation of the diselenide from the GSH-selenenyl sulfide of ebselen by $L(SH)_2$ was also determined. It was found that immediately after the addition of $L(SH)_2$ the diselenide was formed (Fig. 9). $L(SH)_2$ was far more effective than GSH in the generation of the diselenide from the GSH-selenenyl sulfide of ebselen (Figs. 5 and 6). GSH does not affect the $L(SH)_2$-mediated peroxidase activity of ebselen, because the GSH-selenenyl sulfide of ebselen is rapidly converted into the diselenide by $L(SH)_2$. In principle, there are two possible nucleophilic substitution mechanisms for the reaction between $L(SH)_2$ and the GSH-selenenyl sulfide of ebselen (Fig. 13). Firstly, by an attack at the selenium atom, $L(SH)_2$ might substitute for GSH in the GSH-selenenyl sulfide of ebselen (Fig. 13, *reaction 2*^{a}). The formed $L(SH)_2$-selenenyl sulfide of ebselen is rapidly converted into the selenol intermediate (Fig. 13, *reaction 2*^{b}). Secondly, $L(SH)_2$ may attack at the sulfur atom of the GSH-selenenyl sulfide (Fig. 13, *reaction 1*). In that case, the selenol and a mixed disulfide of GSH with $L(SH)_2$ would be formed. The mixed disulfide is probably rapidly converted into lipoate and GSH (13). Both nucleophilic substitutions give rise to a selenol and, after a nucleophilic substitution of the selenol at the selenium of a second GSH-selenenyl sulfide of ebselen, the diselenide is formed.

In order to determine which mechanism depicted in Fig. 13 is favored, it can be argued that a selenol anion is a better

[1] Unpublished results indicate that the pK_a for both thiol groups, when the other thiol group is not deprotonated, is 8.4.

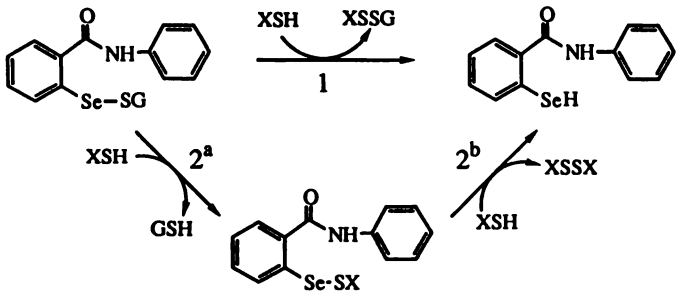

Fig. 13. Possible reactions of thiols (*XSH*) with the GSH-selenenyl sulfide of ebselen. Thiols may attack at the sulfur atom of the GSH-selenenyl sulfide and directly form a selenol (*reaction 1*). Alternatively, thiols may attack at the selenium atom of the GSH-selenenyl sulfide (*reaction 2*^{a}). The formed XSH-selenenyl sulfide can be converted into a selenol by an attack of a thiol at the sulfur of the formed selenenyl sulfide (*reaction 2*^{b}). For MSH, reactions 1 and 2^b proceed slow, while reaction 2^a is fast. For $L(SH)_2$, reaction 2^b is fast, and selenol formation from the GSH-selenenyl sulfide of ebselen is also fast. The latter effect is probably due to a fast thiol exchange of $L(SH)_2$ with GSH in the GSH-selenenyl sulfide, comparable to MSH, and subsequent fast selenol formation from the $L(SH)_2$-selenenyl sulfide of ebselen (reaction 2^a plus 2^b).

leaving group, compared with a thiolate anion; however, nucleophilic substitution at dicoordinate selenium is much faster than nucleophilic substitution at dicoordinate sulfur (26). The latter difference between selenium and sulfur is also illustrated by the fact that the thiols did not react with PZ 25, the sulfur analog of ebselen, whereas the reaction of the thiols with ebselen proved to be fast (Fig. 5). For bis-alkylthio-selenides, the two opposite factors in the reactivity of selenium compared with the reactivity of sulfur appear to cancel each other out. In this case a factor like the steric bulk of the alkyl groups determines whether the sulfur or the selenium is attacked (26). To determine which mechanism prevails in the case of $L(SH)_2$ and the GSH-selenenyl sulfide of ebselen, MSH was used as a tool.

Like GSH, MSH was found to react rapidly with ebselen to form a selenenyl sulfide. Diselenide formation depended on the concentration of free MSH and the second-order rate constant for diselenide formation with MSH (2.2 ± 0.1 mM^{-1} min^{-1}) was lower than with GSH (6.1 ± 0.4 mM^{-1} min^{-1}). In contrast to the $L(SH)_2$-selenenyl sulfide of ebselen, the MSH-selenenyl sulfide of ebselen is stable. Therefore, the reaction between MSH and the GSH-selenenyl sulfide of ebselen can be used to elucidate the mechanism of the reaction between $L(SH)_2$ and the GSH-selenenyl sulfide of ebselen.

When ebselen, MSH, and GSH were present at equimolar concentrations, the selenenyl sulfides of both thiols were detected. The ratio of the concentration of the MSH-selenenyl sulfide to the concentration of the GSH-selenenyl sulfide proved to be independent of the sequence of addition (Fig. 10). This indicates that virtually immediately both selenenyl sulfides are in equilibrium. So, the nucleophilic substitution on the selenium of the selenenyl sulfide was fast for both thiols tested. In equilibrium, approximately half of the selenenyl sulfide consisted of the MSH-selenenyl sulfide of ebselen, indicating that MSH was equally effective as a nucleophile at the selenium of the GSH-selenenyl sulfide as GSH at the selenium of the MSH-selenenyl sulfide. The fact that GSH was a better nucleophile than MSH [the pK_a of the sulfhydryl group of GSH (pK_a = 8.6–8.9) is lower than the pK_a of MSH (pK_a = 9.6) (19)] is probably balanced out by the fact that GSH is also a better leaving group than MSH.

With GSH or MSH, diselenide formation from ebselen was slow with either one of the thiols alone or with the combination of both. With GSH (100 μM) alone, the second-order rate constant was higher than with MSH (100 μM) alone. Apparently, GSH is a better nucleophile at the sulfur of the GSH-selenenyl sulfide than MSH is at the sulfur of the MSH-selenenyl sulfide. The difference between GSH and MSH in diselenide formation can be explained by reasoning that GSH was probably the better nucleophile under the incubation conditions used and that the sulfur of the GSH-selenenyl sulfide might be a better electrophile. In the reaction of either GSH or MSH at the sulfur of their corresponding selenenyl sulfide, the leaving group is identical, namely the selenol.

In the reaction between MSH and the GSH-selenenyl sulfide of ebselen, exchange of thiols is fast while diselenide formation is slow (Fig. 10). This indicates that MSH reacts primarily at the selenium of the GSH-selenenyl sulfide of ebselen (Fig. 13, *reaction 2*^{a}). When these results are extrapolated to the reaction between $L(SH)_2$ and the GSH-selenenyl sulfide of ebselen, the first step is probably the nucleophilic substitution of GSH by $L(SH)_2$ on the selenium (Fig. 13, *reaction 2*^{a}). The formed $L(SH)_2$-selenenyl sulfide of ebselen rapidly forms a selenol (Fig. 13, *reaction 2*^{b}). Diselenide formation finally proceeds by a nucleophilic substitution by the selenol at the selenium of another GSH-selenenyl sulfide. The proposed scheme for the reaction of $L(SH)_2$ with the GSH-selenenyl sulfide of ebselen implies that $L(SH)_2$ is a better cofactor than GSH in the peroxidase activity of ebselen, not because $L(SH)_2$ is a better reducing agent than GSH (in that case, *reaction 1* in Fig. 13 would be preferred), but because of the vicinal thiol groups in $L(SH)_2$ that can form an intramolecular disulfide.

In vivo, more GSH than $L(SH)_2$ is available (19). However, as shown in this study, in the peroxidase activity of ebselen, $L(SH)_2$, at a concentration of 5 μM, was found to be more potent than GSH in the millimolar range (Figs. 2 and 3). Moreover, GSH does not reduce the $L(SH)_2$-mediated peroxidase activity of ebselen. It should be noted however, that *in vivo* most $L(SH)_2$ is bound in an amide linkage to the ϵ-amino group of a lysine residue. In its biological function $L(SH)_2$ shuttles between the oxidized and reduced form (27). In the reaction of bound lipoate with pyruvate, first pyruvate is cleaved by a carboxylase to yield CO_2 and an enzyme-bound "active aldehyde." Subsequently the active aldehyde is believed to attack the disulfide linkage of bound lipoic acid in a nucleophilic displacement reaction, followed by a reverse condensation (27). It is presumed that the acetyl group is attached to the primary SH group of bound $L(SH)_2$ (24). The acetyl group is transferred from $L(SH)_2$ to coenzyme A, a reaction catalyzed by lipoic acid transacetylase. Finally, $L(SH)_2$ reduces NAD^+ to NADH and lipoate. The latter reaction is catalyzed by a dihydrolipoic dehydrogenase, and it has been reported that this reaction is freely reversible with high reaction rates in both directions (27). The reaction of pyruvate or NADH with membrane-bound lipoate might provide reducing equivalents for the peroxidase activity of ebselen.

Recently, it has been reported that ebselen totally blocks *in vivo* Sephadex-induced lung edema in rats (28). Coadministration of GSH reduced the protective effect of ebselen. It has been stated that this is due to the formation of the GSH-selenenyl sulfide of ebselen. It was suggested that the formed selenenyl sulfide had a much higher aqueous solubility than

the parent ebselen, and the loss of activity of ebselen was ascribed to the enhanced clearance of ebselen, as selenenyl sulfide, from the animal (28). As demonstrated in this study, $L(SH)_2$ is capable of converting the GSH-selenenyl sulfide of ebselen rapidly into the diselenide (Fig. 9). Inferred from the fact that the retention time of the diselenide is longer than that of ebselen with reverse phase HPLC, the diselenide is probably less hydrophilic than ebselen. Therefore, conversion of ebselen into the diselenide probably will not enhance renal clearance. As already mentioned, $L(SH)_2$ is a better cofactor than GSH in the peroxidase activity of ebselen. Additionally, $L(SH)_2$ might have an important contribution in the therapeutic effect of ebselen by preventing the enhanced clearance of ebselen as GSH-selenenyl sulfide.

Acknowledgments

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Appendix

The derivation of the equation that describes the GSH peroxidase activity of ebselen, according to the mechanism depicted in Fig. 11, is shown. The derivation is based on the method used to describe the peroxidase activity of the selenium-dependent GSH peroxidase, according to Flohé *et al.* (7). The abbreviations used are: *A*, ebselen; *B*, ebselen-GSH adduct; *C*, selenol; *D*, diselenide; *E*, selenenic acid anhydride; *T*, total concentration of ebsenen added; *V*, peroxidase activity.

The reactions are:

$$A + \mathrm{GSH} \xrightarrow{k_1} B \quad \text{(I)}$$

$$B + \mathrm{GSH} \xrightarrow{k_2} C + \mathrm{GSSG} \quad \text{(II)}$$

$$C + B \xrightarrow{k_3} D + \mathrm{GSH} \quad \text{(III)}$$

$$D + \mathrm{H_2O_2} \xrightarrow{k_4} E + \mathrm{H_2O} \quad \text{(IV)}$$

$$E \xrightarrow{k_5} 2A + \mathrm{H_2O} \quad \text{(V)}$$

Based on this reaction scheme, the following equations can be derived:

$$\frac{d[A]}{dt} = 2k_5[E] - k_1[\mathrm{GSH}][A] \quad (1)$$

$$\frac{d[B]}{dt} = k_1[\mathrm{GSH}][A] - k_2[\mathrm{GSH}][B] - k_3[B][C] \quad (2)$$

$$\frac{d[C]}{dt} = k_2[\mathrm{GSH}][B] - k_3[B][C] \quad (3)$$

$$\frac{d[E]}{dt} = k_4[\mathrm{H_2O_2}][D] - k_5[E] \quad (4)$$

The concentration of ebselen added (T) is:

$$T = [A] + [B] + [C] + 2[D] + 2[E] \quad (5)$$

The peroxidase activity (V) is:

$$V = \frac{-d[\mathrm{H_2O_2}]}{dt} = k_4[\mathrm{H_2O_2}][D] \quad (6)$$

In steady state conditions:

$$\frac{d[A]}{dt} = \frac{d[B]}{dt} = \frac{d[C]}{dt} = \frac{2d[D]}{dt} = \frac{2d[E]}{dt} = 0 \quad (7)$$

Combination of Eqs. 4 and 7 gives:

$$[E] = \frac{k_4[\mathrm{H_2O_2}]}{k_5}[D] \quad (8)$$

Combination of Eqs. 1, 7, and 8 gives:

$$[A] = \frac{2k_5}{k_1[\mathrm{GSH}]}[E] = \frac{2k_4[\mathrm{H_2O_2}]}{k_1[\mathrm{GSH}]}[D] \quad (9)$$

Combination of Eqs. 2, 7, 3, and 9 gives:

$$[B] = \frac{k_1}{2k_2}[A] = \frac{k_4[\mathrm{H_2O_2}]}{k_2[\mathrm{GSH}]}[D] \quad (10)$$

Combination of Eqs. 3 and 7 gives:

$$[C] = \frac{k_2}{k_3}[\mathrm{GSH}] \quad (11)$$

Substitution of Eqs. 8–11 in Eq. 5 gives:

$$T = \left\{\frac{2k_4[\mathrm{H_2O_2}]}{k_1[\mathrm{GSH}]} + \frac{k_4[\mathrm{H_2O_2}]}{k_2[\mathrm{GSH}]} + 2 + \frac{2k_4[\mathrm{H_2O_2}]}{k_5}\right\}[D] + \frac{k_2[\mathrm{GSH}]}{k_3} \quad (12)$$

Substitution of Eq. 12 in Eq. 6 gives:

$$V = k_4[\mathrm{H_2O_2}]\left\{\frac{2k_4[\mathrm{H_2O_2}]}{k_1[\mathrm{GSH}]} + \frac{k_4[\mathrm{H_2O_2}]}{k_2[\mathrm{GSH}]} + 2 + \frac{2k_4[\mathrm{H_2O_2}]}{k_5}\right\}^{-1}\left\{T - \frac{k_2[\mathrm{GSH}]}{k_3}\right\} \quad (13)$$

Eq. 13 can be simplified to:

$$V = \left\{\frac{2}{k_1[\mathrm{GSH}]} + \frac{1}{k_2[\mathrm{GSH}]} + \frac{2}{k_4[\mathrm{H_2O_2}]} + \frac{2}{k_5}\right\}^{-1}\left\{T - \frac{k_2[\mathrm{GSH}]}{k_3}\right\}$$

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