The antioxidant mechanism of ebselen involves recently discovered reductions by mammalian thioredoxin reductase (TrxR) and thioredoxin (Trx) forming ebselen selenol. Here we describe a previously unknown reaction; ebselen reacts with its selenol forming an ebselen diselenide with a rate constant of 372 M⁻¹s⁻¹. The diselenide also was a substrate of TrxR forming the selenol with $K_m$ of 40 μM and $k_{cat}$ of 79 min⁻¹ ($k_{cat}/K_m$ of $3.3 \times 10^4$ M⁻¹s⁻¹). Trx increased the reduction because of its fast reaction with diselenide (rate constant 1.7 $\times$ 10³ M⁻¹s⁻¹). Diselenide stimulated the H₂O₂ reductase activity of TrxR, even more efficiently with Trx present. Because the mechanism of ebselen as an antioxidant has been assumed to involve glutathione peroxidase-like activity, we compared the H₂O₂ reductase activity of ebselen with the GSH and Trx systems. TrxR at 50 nM, far below the estimated physiological level, gave 8-fold higher activity compared with 1 mM GSH; addition of 5 μM Trx increased this difference to 13-fold. The rate constant of ebselen selenol reacting with H₂O₂ was estimated to be faster than 350 M⁻¹s⁻¹. We propose novel mechanisms for ebselen antioxidant action involving ebselen selenol and diselenide formation, with the thioredoxin system rather than glutathione as the predominant effector and target.

Ebselen¹ (2-phenyl-1,2-benzisoselenazol-3(2H)-one, also called PZ51) is a seleno-organic compound, originally synthesized while searching for compounds that mimic the activity of the endogenous antioxidant glutathione peroxidase (1). Numerous studies have provided a wealth of information that this compound has anti-inflammatory, antiatherosclerotic, and cytoprotective properties in both in vitro and in vivo models (2–6). Unlike other selenium compounds, ebselen possesses a very low toxicity because of its unique stability in structure and because its selenium moiety is not liberated during biotransformation and therefore does not enter the selenium metabolism of the organism (7–9). In more recent animal model studies, ebselen was shown to reduce oxidative stress in ischemia-reperfusion in heart (10) and to have neuroprotective effects in brain (11–14). More importantly, ebselen has been demonstrated to have beneficial effects in clinical trials for the treatment of patients with delayed neurological deficits after aneurysmal subarachnoid hemorrhage (15) and acute ischemic stroke (16, 17).

The thioredoxin system, a collective name for thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH, is the most powerful protein disulfide reductase system in cells and is present in all living organisms (18–21). TrxR is a dimeric FAD-containing enzyme, which catalyzes the NADPH-dependent reduction of the active site disulfide in oxidized Trx (Trx-S₂) to give a dithiol in reduced Trx (Trx-(SH)₂) (18, 19). The reduced Trx is a hydrogen donor for ribonucleotide reductase, the essential enzyme for DNA synthesis and a powerful general protein disulfide reductase with a large number of functions in growth and redox regulations (18–21). As an endogenous antioxidant, the thioredoxin system serves to keep the redox state of a cell balanced (18–21).

Mammalian TrxRs are large selenoproteins (Mᵣ, 114,000 or larger) with structures showing a close homology to glutathione reductase but with an elongation containing a unique catalytically active selenolthiol/selenenylsulfide in the conserved C-terminal sequence Gly-Cys-Sec-Gly (22–27). Mammalian TrxRs have a remarkably wide substrate specificity (18–22), reducing not only different thioredoxins but also e.g. selenite (28), selenodiglutathione (29), and selenocystine (30). The mammalian enzymes also are NADPH-dependent lipid hydroperoxide reductases (31) and may serve directly as an electron donor of human plasma glutathione peroxidase (32).

In a previous study, we reported that ebselen is a substrate of mammalian TrxR, which is reduced by NADPH forming ebselen selenol with a $K_m$ value of 2.5 μM and a $k_{cat}$ of 588 min⁻¹ (33). We also showed that ebselen strongly enhanced the hydrogen peroxide reductase activity of mammalian TrxR, acting as a TrxR peroxidase. In the presence of Trx, ebselen acted as a mimic of a peroxiredoxin (33). In reduction of ebselen by mammalian TrxR, we observed that under certain conditions, ebselen reacted with its selenol forming ebselen diselenide (2,2-diselenobis-(N-phenyl)-benzamid in Reaction 1), which absorbs strongly at 340 nm and has a low solubility giving rise to a precipitate and increase in $A_{340}$ (33).
Here we report a study on the interaction of ebselen diselenide with the mammalian thioredoxin system to clarify the role of this compound in the H$_2$O$_2$ reductase activity of ebselen. We found that ebselen diselenide also is a direct substrate of mammalian TrxR and that Trx increases the reduction rate. The kinetics of the formation of ebselen diselenide (Reaction 1) from ebselen reacting with its selenol was measured.

Ebselen has been widely used as an antioxidant in experimental models assuming that it is acting via a GSH peroxidase-like mechanism (1–6). The discovery of its reactivity with thioredoxin reductase and thioredoxin (33) changed this picture. Using glutathione as the reductant, the H$_2$O$_2$ reductase activity of ebselen was compared with that in the presence of the mammalian thioredoxin system. Our results demonstrate that ebselen uses the thioredoxin system far more efficiently than glutathione. Formation of ebselen diselenide may serve as a dose-dependent storage form of ebselen, which can be relatively slowly activated to the catalytically active selenol by the mammalian thioredoxin system.

EXPERIMENTAL PROCEDURES

Materials and Enzymes—NADPH, DTT, and DTNB were from Sigma. Hydrogen peroxide (perhydrol) (30%) and dimethyl sulfoxide (Me$_2$SO) were from Merck. TrxR from calf thymus or human placenta was purified to homogeneity (25) essentially as described for the rat liver enzyme (20, 22). Trx from *Escherichia coli* was a homogeneous preparation, and recombinant human Trx and the mutant C61S/C72S prepared as described by Ren et al. (34) were from IMCO Ltd, Stockholm, Sweden (www.imcocorp.se). The sources of other materials have been described in previous publications (25, 28, 29). Ebselen and ebselen diselenide were products of Daiichi and were dissolved in fresh Me$_2$SO before addition into the samples. Concentrations of Me$_2$SO were less than 5% of the solvent buffer, effective in dissolved the drug.

Enzyme Assays—The activity of enzymes was determined at room temperature using a Uvikon 3000 UV/visible spectrophotometer (Amersham Biosciences). Measurements of TrxR activity were performed in a buffer containing 50 mM Tris-Cl, 1 mM EDTA, pH 7.5, generally with 100 \mu M NADPH and the indicated amounts of ebselen. Reactions were started with addition of 5 or 10 \mu l of a stock solution of TrxR in a final total volume of 0.50 ml. Cuvettes containing reference mixtures contained the same amount of Me$_2$SO in the samples and TrxR.

The glutathione peroxidase activity of ebselen was measured according to Wilson’s method (35). The reactions were carried out at 37 °C in 0.5 ml of solution containing 50 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 1 mM sodium azide, 1 mM GSH, 1 unit of GSH reductase, and 2 \mu M ebselen. The mixtures were preincubated for 10 min, and NADPH was added to a final concentration of 250 \mu M. The reactions were then initiated by addition of 0.5 mM H$_2$O$_2$. The activities were followed by the decrease of NADPH at 340 nm against blanks without ebselen.

Fluorescence Measurement—Protein fluorescence was measured with a thermostatic SPEX-FluroMax Spectrofluorometer. Trx-(SH)$_2$ was prepared from *E. coli* Trx-S$_2$, which was incubated at room temperature for 20 min with 10 mM DTT. DTT was subsequently removed by gel chromatography on a NAP-5 column (Amersham Biosciences) using N$_2$-equilibrated buffer. Trx-(SH)$_2$ was mixed with ebselen in a total volume of 3 ml of potassium phosphate, 1 mM EDTA, pH 7.5 directly at 22 °C. Excitation of fluorescence was at 290 nm and emission spectra from 300 to 500 nm were recorded. Emission at 340 nm was followed to record the rate of oxidation of Trx-(SH)$_2$ by ebselen diselenide (18, 26).

RESULTS

Absorption Spectrum of Ebselen Diselenide—Using the authentic compound, the absorption spectrum of 30 \mu M ebselen diselenide in TE buffer, pH 7.5, the spectrum of 30 \mu M ebselen diselenide was measured in a 1-cm quartz cuvette.

**Fig. 1. An absorption spectrum of ebselen diselenide.** In TE buffer containing 2% Me$_2$SO, pH 7.5, the spectrum of 30 \mu M ebselen diselenide was measured in a 1-cm quartz cuvette.

**Results**

**Enzymatic Models**—Ebselen diselenide was measured in a 1-cm quartz cuvette. The extinction coefficient of (EbSe)$_2$ at 340 nm ($\varepsilon_{340}$) was calculated to be 21,000 M$^{-1}$cm$^{-1}$ using several known concentrations of (EbSe)$_2$. It should be noted that most assays of ebselen and its reactivity toward hydroperoxides were measured at 340 nm, where the kinetic oxidation of NADPH to NADP$^+$ was followed. The high and broad absorption of (EbSe)$_2$ has to be taken into consideration when a decrease of $A_{340}$ is interpreted. Another useful wavelength for (EbSe)$_2$ measurement is 390 nm with $\varepsilon_{390}$ of 8000 M$^{-1}$cm$^{-1}$, where most of the other substances used in the assays have no or very weak absorbance.

**Ebselen Reacts with Its Selenol to Form an Ebselen Diselenide**—Reduction of ebselen by NADPH catalyzed by mammalian TrxR produces ebselen selenol (33). However, when lower concentrations of the enzyme and higher concentrations of ebselen were used, the A$^{340}$ showed a complex change. Fig. 2 shows the change of A$^{340}$ upon the addition of 10–100 \mu M ebselen by NADPH catalyzed by 7.5 \mu M TrxR. With higher concentrations of ebselen (>20 \mu M), the A$^{340}$ first increased, followed by a decrease to give the final reduction product ebselen selenol. The highest concentrations (50 and 100 \mu M) gave visible precipitates. This is due to a fast reaction of excess ebselen with its reduction product ebselen selenol through Reaction 1.

The rate constant of Reaction 1 was measured in a way where 40 \mu M ebselen selenol is produced by reduction of 40 \mu M ebselen with 100 \mu M NADPH catalyzed by 50 nm calf liver TrxR (Fig. 3A). After 20 min, where the reaction was complete, the enzyme in the solution was removed by filtering though an Ultrafree-MC Millipore 10,000 cutoff filter. To this solution containing 40 \mu M ebselen selenol, another 40 \mu M ebselen was added, and the kinetics of formation of (EbSe)$_2$ was followed at 390 nm (Fig. 3B). The formation of ebselen diselenide was fitted to second-order rate equation 1, where $x$ is the micromolar concentration of (EbSe)$_2$, using $\varepsilon_{390}$ of 8000 M$^{-1}$s$^{-1}$, and $a$ is the initial concentration of ebselen and ebselen selenol, in this case, 40 \mu M.

$$\frac{x/a-a-x}{a-x} = k_1 \cdot t$$  \hspace{1cm} (Eq. 1)
As seen from the inset of Fig. 3B, a plot of the left side of Equation 1 against time gave a straight line confirming second-order kinetics with a slope corresponding to the second-order rate constant $k_1$ of 0.0223 M$^{-1}$s$^{-1}$.

Ebselen Diselenide Is a Substrate of Mammalian TrxR, and Human Trx Increases the Reaction Rate—To TE buffer solutions (pH 7.5) containing 10 and 20 mM ebselen diselenide, 100 mM NADPH, 50 nM calf thymus TrxR were added. The decreases of (EbSe)$_2$ were followed against an identical blank without TrxR (Fig. 4). Ebselen diselenide was a direct substrate of mammalian TrxR, and addition of human Trx increased the reaction rate. Reduction of ebselen diselenide by NADPH catalyzed by TrxR produced ebselen selenol (Reaction 2), as evidenced by the final spectrum and HPLC analysis (data not shown). Addition of one volume of 6 M guanidine hydrochloride containing 10 mM DTNB at the end of the reaction also gave formation of TNB, showing the reduction of DTNB by the ebselen selenol in Reaction 2.

The steady state kinetics of ebselen diselenide as a substrate of TrxR was also measured directly using 100 nM mammalian TrxR. As shown in Fig. 5, the $K_m$ value of 40 $\mu$M and $k_{cat}$ of 79 min$^{-1}$ can be derived from a Lineweaver-Burk Plot of an assay, and the $k_{cat}/K_m$ was calculated to be $3.3 \times 10^4$ M$^{-1}$s$^{-1}$.

Reduction of Ebselen Diselenide by E. coli Trx and DTT—From Fig. 4, it was obvious that addition of Trx to TrxR increased reduction rate of NADPH toward ebselen diselenide. This indicated that Trx-(SH)$_2$ is a fast reductant of (EbSe)$_2$ according to Reactions 3 and 4 in the following reaction scheme.

Mammalian and E. coli Trx have the same active site (WCGPC) and reactivity with disulfides (18, 26). Since E. coli Trx-(SH)$_2$ has a 3-fold higher tryptophan fluorescence than TrxS$_2$ (18, 26), this was used to follow the reaction with ebselen diselenide. The fluorescence decay of 5 $\mu$M reduced E. coli Trx-(SH)$_2$ was recorded after adding 5 $\mu$M (EbSe)$_2$ (Fig. 6). The inverse of the Trx-(SH)$_2$ fluorescence intensity was found to be linear against time indicating a typical second-order kinetics (Fig. 6, inset). Thus the slope of the linear fit gave a second-
Ebselen Diselenide Stimulates the H$_2$O$_2$ Reductase Activity of Mammalian TrxR—Because reduction of ebselen diselenide by NADPH produces two equivalents of ebselen selenol, which will rapidly reduce H$_2$O$_2$ and generate ebselen, a much more efficient substrate of mammalian TrxR and thus a TrxR peroxidase mimic (33), ebselen diselenide should also stimulate the H$_2$O$_2$ reductase activity of mammalian TrxR. Fig. 7 showed that the changes in $A_{340}$ as recorded after addition of 1–10 $\mu$M (EbSe)$_2$ to TE buffers, pH 7.5, containing 250 $\mu$M NADPH, 18 nM mammalian TrxR, and 0.5 mM H$_2$O$_2$. These H$_2$O$_2$ reductase activities of (EbSe)$_2$ were compared with that of 2 $\mu$M ebselen (dark line). Obviously (EbSe)$_2$ stimulated the H$_2$O$_2$ reductase activity of TrxR. However, we also noticed that this activity was higher than expected, since ebselen diselenide is about a 100-fold less efficient substrate of the TrxR than ebselen, as we have described above. Although we cannot account for the high reactivity of ebselen diselenide, which may have to do with a direct interaction of the oxidized form of ebselen diselenide with the thiorodixin system. At higher (EbSe)$_2$ concentrations, the activity as a glutathione peroxidase mimic, catalyzing the glutathione detoxification of hydroperoxides (1–4). A number of other thios, e.g. N-acetyl-L-cysteine (40), dithiothreitol (41), and dihydrolipoate (42) were also used. The endogenous dihydrolipoate was found to be a better cofactor than glutathione for the peroxidase activity of ebselen (42).

We compared the H$_2$O$_2$ reductase activities of ebselen using a lower than normal physiological concentrations of TrxR (50
Even larger since with 50 nM TrxR and 0.5 mM H9262, the rate of NADPH has not reached saturation and then the concentration of NADPH should be much lower than 2 mM. Thus, the increase of the H2O2 reductase activity of ebselen to 13-fold. Reaction 1 was not recognized. The occurrence of the selenenylsulfide to (EbSe)2 by thiols was clearly demonstrated by Cotgreave et al. (51) by trapping the selenol with 1-chloro-2,4-dinitrobenzene. Formation of diselenide of ebselen was evident from both HPLC and 77Se NMR analysis (42). However, the mechanistic explanations of the chemical conversions of ebselen reacting with thiols were controversial (42, 52), and Reaction 1 was not recognized. The

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argument also included whether the active form of ebselen toward H₂O₂ is ebselen diselenide (42, 52) or the selenol (46, 50). Formation of a diselenide was suggested to be a key step for catalytic activity as the slowest one, and its reaction with the H₂O₂ would yield the parent compound and water (42, 52). However, Maiorino et al. (50) concluded that the ebselen mechanism appeared kinetically identical to the enzyme reaction and showed that carboxymethylation of intermediates by iodoacetamide formed inactive derivatives suggested that an ebselen selenol is involved. Morgenstern et al. (46) also concluded that the selenol is the predominant molecular species responsible for the GSH-dependent peroxidase activity of ebselen. Because we have a reaction system where ebselen directly undergoes a fast two-electron reduction by NADPH catalyzed by mammalian TrxR, forming the ebselen selenol, the occurrence of Reaction 1 became evident. The formation of the ebselen diselenide, measured to have a second order rate constant of 372 M⁻¹ s⁻¹, was apparent when both ebselen and ebselen selenol are present, e.g. in reduction reactions at high concentrations of ebselen and low concentrations of enzymes.

Formation of the diselenide was not a unique feature of the enzyme since it could be mimicked by using a low non-stoichiometric concentration of DTT (10 μM) and 100 μM ebselen, whereas excess DTT only gave the selenol as is also shown by HPLC (data not shown). It is therefore not surprising that dithiols like DTT or dihydrolipoate are better cofactors of ebselen than glutathione as observed (41, 42). When the mammalian thioredoxin system was used, ebselen was highly efficiently reduced to its selenol, which in the presence of H₂O₂, rapidly forms H₂O and a selenenic acid, and then spontaneously eliminates H₂O and regenerates the ebselen for another catalytic cycle (Reaction 6).

We have clearly shown that ebselen diselenide is also a substrate of mammalian TrxR with a Kₘ value of 40 μM and kₐₑₜ of 79 min⁻¹; thus the kₐₑₜ/Kₘ is calculated to be 3.3 × 10⁶ M⁻¹ s⁻¹. Compared with the reduction of ebselen by NADPH catalyzed by mammalian TrxR having a Kₘ value of 2.5 μM, a kₐₑₜ of 588 min⁻¹ and a kₐₑₜ/Kₘ of 3.9 × 10⁵ M⁻¹ s⁻¹ (33), the efficiency of ebselen diselenide reduction is 100 times slower. The mechanism of mammalian TrxR acting on ebselen diselenide can be illustrated in Scheme 2. The C-terminal active site selenenylsulfide in TrxR is reduced by NADPH via the active site disulfide in the second subunit forming a thiol and a selenolate (25–27, 53). The latter will reduce (EbSe)₂ via a short-lived intermediate, TrxRSe-SeEbSe, and will form two equivalents of ebselen selenol. Ebselen diselenide also was shown to have an H₂O₂ reductase activity (Fig. 7), which was surprisingly high compared with that of ebselen, taking into account the difference as the substrates for the enzyme as described above. A simple mechanism for (EbSe)₂ acting as an H₂O₂ reductase is given in Scheme 3. Reduction of (EbSe)₂ by the mammalian TrxR slowly forms the ebselen selenol, which will rapidly reduce H₂O₂ and generate ebselen, a 100-fold more efficient substrate of mammalian Trx system. At the same time, ebselen will also react with the ebselen selenol to reform the ebselen diselenide through Reaction 1. Thus the H₂O₂ reductase activity of (EbSe)₂ is actually achieved through a bridge connecting the active form of the catalytic cycle, i.e. the ebselen selenol and ebselen, with H₂O₂. The pathways described in Scheme 3 also stand for a complete mechanism of ebselen for its H₂O₂ reductase activity. A more simplified form of Scheme 3 is drawn in Scheme 4, including a previously unrecognized part of the antioxidant mechanism of ebselen, i.e. the ebselen itself will compete with H₂O₂ for the ebselen sel-
enol to form the ebselen diselenide, which in fact limits the level of its $\text{H}_2\text{O}_2$ reductase activity. Since the reduction of (EhSe)$_2$ by NADPH catalyzed by the mammalian Trx system is much slower than that of ebselen, (EhSe)$_2$ may then serve as a storage form of ebselen, which is slowly reactivated by the mammalian Trx system. Formation of the diselenide is definitively a part of ebselen antioxidant action, which will affect its efficiency as a peroxidase but will not change the character of ebselen as a thioredoxin reductase and thioredoxin peroxidase mimic.

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