The Reaction of Ebselen with Peroxynitrite

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Ebselen, 2-phenyl-1,2-benzisoselenazol-3(*2H*)-one, rapidly reacts with peroxynitrite, the rate constant being of the order of 10^6 M^{-1} s^{-1}; the reaction yields the selenoxide of the parent molecule, 2-phenyl-1,2-benzisoselenazol-3(*2H*)-one 1-oxide, as the sole selenium-containing product; a stoichiometry of 1 mol of ebselen reacted and of the selenoxide formed per mole of peroxynitrite was observed. The reaction was studied in detail at neutral and alkaline pH (pH 10−11). It also proceeds at acidic pH where peroxynitrous acid (ONOOH) is predominant, the yield of the selenoxide being lower because peroxynitrous acid ($pK_a = 6.8$) decays rapidly. Reduction of the selenoxide in cells to regenerate ebselen would allow for a sustained defense against peroxynitrite. This novel reaction constitutes a potential cellular defense line against peroxynitrite, one of the important reactive species in inflammatory processes.

Introduction

Peroxynitrite[1] is one of the reactive species generated in the inflammatory process, e.g., by the reaction of the superoxide anion radical ($O_2^{\bullet-}$)[2] and nitric oxide ($^\bullet$NO) (*1*, *2*). Superoxide ($O_2^{\bullet-}$) and $^\bullet$NO are generated upon activation of the NADPH oxidase and NO synthase enzymes, respectively, which are present in macrophages and other cell types. Peroxynitrite can undergo a number of reactions of pharmacological and toxicological interest (*1*, *3*, *4*). It initiates lipid peroxidation, oxidizes sulfhydryls at a substantially faster rate than its first-order decay (*5*), reacts with hydrogen peroxide to form singlet molecular oxygen (*6*), and reacts with methionine (*7*, *8*) and ascorbic acid (*9*). Also it nitrates and hydroxylates aromatic compounds (*10*, *11*) and leads to the nitration of protein tyrosines (*12*).

The peroxynitrite anion ($ONOO^-$, **1**) is relatively stable in alkaline solution, and its conjugate acid, peroxynitrous acid (ONOOH, **1a**), with $pK_a = 6.8$ (*13*) can isomerize to nitrate (the apparent half-life of peroxynitrite, $t_{1/2}$, is 0.1−1 s at pH 7.4 and 37 °C (*1*, *13*, *14*)). Peroxynitrous acid (**1a**) has been suggested to decompose by homolytic scission into nitrogen dioxide ($^\bullet NO_2$) and hydroxyl radical ($^\bullet$OH), and **1a** generates a strong oxidant with reactivity similar to that of the hydroxyl radical (*1*, *15*). Peroxynitrite is thought to play an important role in a potential pathway of tissue damage, but there is no known endogenous defense line against its toxicity, except for the control of the two enzymes mentioned above and the repair activities operative after damage has occurred.

In previous work, we noted that peroxynitrite-induced luminol chemiluminescence emitted from Kupffer cells (*16*) was inhibited in the presence of low concentrations of ebselen (**2**) (*17*). These observations suggested that ebselen (**2**) reacts with peroxynitrite and prompted us to investigate the reaction further.

In the present work, we describe the reactivity of the selenoorganic compound ebselen (**2**) with peroxynitrite. Ebselen (**2**) has been identified as an antiinflammatory agent (*18*−*22*), and it exhibits glutathione peroxidase (GSH-Px) activity which may explain some of its actions (*23*−*26*).

Materials and Methods

Reagents. Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one (**2**), was a kind gift from Rhône-Poulenc-Nattermann, Cologne, FRG. Chemicals, solvents, and silica gel TLC plate (silica gel 60 F_{254}, 20 × 20 cm, 0.25 mm thickness) were from E. Merck, Darmstadt, FRG.

Peroxynitrite was synthesized from sodium nitrite and hydrogen peroxide (H_2O_2) using a quenched-flow reactor as previously described (*1*, *6*). Residual H_2O_2 was eliminated by passage of the peroxynitrite solution over MnO_2 powder. Peroxynitrite concentration was determined spectrophotometrically at 302 nm ($\epsilon = 1670$ $M^{-1}\cdot cm^{-1}$). Peroxynitrite was also synthesized by the autoxidation of hydroxylamine in 0.5 M aqueous NaOH solution (*9*, *27*). Oxygen gas was bubbled at a flow rate of ca. 0.2 L/min into a solution (200 mL) of 10 mM NH_2OH in 0.5 M aqueous NaOH containing 100 μM diethylenetriaminepentaacetic acid (DTPA) for 3 h at room temperature. The resulting solution was treated with granular MnO_2 as described above and filtered. Peroxynitrite was concentrated by freeze fractionation.

Ebselen Se-oxide, 2-phenyl-1,2-benzisoselenazol-3(*2H*)-one 1-oxide (**3**), was synthesized from ebselen (**2**) and H_2O_2 as previously described (*28*). The structure was confirmed by IR, MS, ^{1}H NMR (500 MHz) spectra, and elemental analysis. Small signals which correspond to the hydrolyzed ring-opened form of ebselen Se-oxide (**3**), 2-(phenylcarbamoyl)phenylseleninic acid (**3a**, Se(O)OH), were observed in the ^{1}H NMR spectrum (DMSO-d_6/TMS). These signals were significantly increased after addition of H_2O (1 drop) to the sample solution, and the mixture gave two spots on silica gel TLC tracing (solvent for development: ethyl acetate/methanol/acetic acid, 10/5/1 v/v). Ebselen Se-oxide (**3**) and its hydrolyzed form (**3a**) were found to be in equilibrium.

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[1] Note that the recommended IUPAC nomenclature for peroxynitrite is oxoperoxonitrate; for peroxynitrous acid, hydrogen oxoperoxonitrate; for nitric oxide, nitrogen monoxide. The term peroxynitrite is used in the text to refer generically to both peroxynitrite anion ($ONOO^-$, **1**) and its conjugate acid, peroxynitrous acid (ONOOH, **1a**). When the discussion is limited to either the anion or the acid, its specific name is used.

[2] Abbreviations: $O_2^{\bullet-}$, superoxide anion radical; $^\bullet$NO, nitric oxide (nitrogen monoxide); $ONOO^-$, peroxynitrite anion (oxoperoxonitrate); ONOOH, peroxynitrous acid (hydrogen oxoperoxonitrate); $^\bullet NO_2$, nitric dioxide; $^\bullet$OH, hydroxyl radical; DTPA, diethylenetriaminepentaacetic acid; DMSO, dimethyl sulfoxide; GSH, glutathione; GSH-Px, glutathione peroxidase, SOD, superoxide dismutase.

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General. Prior to experiments, the concentration of the stock peroxynitrite solution was determined by measuring the absorbance at 302 nm of an aliquot of the stock peroxynitrite solution diluted in 0.1 M aqueous NaOH versus an aliquot of the stock solution that was allowed to decompose in 0.5 M sodium phosphate buffer (pH 7.4). The first-order rate constant of spontaneous peroxynitrite decay (k_d) was determined by least-squares fitting of absorbance at 302 nm versus time. Phosphate buffer is the least reactive solvent in terms of accelerating peroxynitrite decomposition (*29*). The buffer was 0.05 or 0.5 M sodium phosphate (pH 10−11) as indicated.

Reaction System. Typically, peroxynitrite diluted in 0.5 M aqueous NaOH was added to a solution of ebselen in sodium phosphate buffer (1.0 mL) while vigorously vortexing to start the reaction at room temperature (22 °C). For HPLC analysis, methanol (2.0 mL) was added to the reaction mixture, and the resulting mixture was cooled to −20 °C to precipitate buffer salts. After centrifugation, the supernatant fraction was separated and stored at −20 °C until analysis. Ebselen (**2**) was used as a solution in methanol or DMSO, and the concentration of organic solvent did not exceed 1% v/v in the reaction mixture. Controls received solvent only. The pH was measured after peroxynitrite addition to account for the slight alkaline shift caused by the NaOH used to stabilize peroxynitrite. Recovery of selenium compounds after the workup procedure was quantitative (>97%).

Alternatively, solutions of peroxynitrite (initial 20 μM) in 0.5 M sodium phosphate buffer (pH 10.2) were prepared. A slight excess amount of **2** (25 μM) was added separately at various times after initial peroxynitrite addition to start the reaction. Reactions were incubated for 10 min at room temperature (22 °C) and then worked up for HPLC as described above.

Spectrophotometric Analysis. Absorbance measurements were performed with a Perkin Elmer Lambda 2 spectrophotometer. Absorbance spectra of the reaction mixture in a quartz cuvette (light path 1 cm) were recorded immediately after addition of peroxynitrite against buffer solution as reference. Difference spectra were recorded against an ebselen reference solution. Ebselen oxidation was measured by the absorbance difference, ΔA (279 − 327 nm).

HPLC Analysis. Aliquots of samples (typically, 20 μL) were injected onto a reversed-phase column (RP C18, 150 mm × 4.6 mm i.d., Capcell Pak C_{18} (SG120) 5 μm; Shiseido Co., Tokyo). Separation was performed with a 10 mM sodium phosphate buffer, (pH 7.4)/acetonitrile gradient on a Merck-Hitachi L-6200A HPLC unit, at a flow rate of 1.0 mL/min. The linear gradient was typically from 80/20 to 24/76 over 20 min. The selenoorganic compounds were monitored with a Merck-Hitachi 655A UV detector equipped with D-2500 Chromato-Integrator at 254 nm. Ebselen Se-oxide (**3**) was fully separated from its parent substrate, ebselen (**2**), and appeared as single peak (see Figure 3). Calibration curves were calculated from peak areas versus concentration values of authentic samples carried through the extraction procedure and HPLC analysis. The calibration curves were used to calculate the concentrations of the compounds in the samples from their peak areas.

Nitrite Quantitation. Nitrite (NO_2^-) concentration was measured by the Griess reaction (*30*) with minor modifications. Sample (1.0 mL) was added to 1% w/v sulfanilamide solution (1.0 mL in 2 M HCl). Thereafter, 1% w/v *N*-(1-naphthyl)-ethylenediamine solution (1.0 mL in 2 M HCl) was added to the mixture. After 10 min at room temperature, the absorbance of the resulting mixture at 540 nm was measured. Calibration curves were calculated from the absorbance of standard $NaNO_2$ solutions.

Results

Stability of Peroxynitrite. Spontaneous decomposition of peroxynitrite in 0.5 M sodium phosphate buffer (pH 10.2) at room temperature (22 °C) was measured by monitoring the absorbance at 302 nm of the peroxynitrite solution. The first-order rate constant of spontaneous peroxynitrite decay (k_d) under these conditions was calculated to be ca. 1×10^{-3} s^{-1} by exponential fitting.

Reactivity of Ebselen with Peroxynitrite. The reaction of ebselen (**2**) with peroxynitrite in 0.05 M sodium phosphate buffer (pH 10.9) was followed spectrophotometrically. The spectrum of **2** (50 μM) with an absorbance maximum at 325 nm was altered substantially after addition of peroxynitrite (30 μM), as shown in Figure 1A. The spectrum of the reaction mixture after addition of a slight excess of peroxynitrite (60 μM) was similar to that of authentic ebselen Se-oxide (**3**) (Figure 1A). Further addition of peroxynitrite had no effect on the spectrum. The spectral changes were very rapid and were complete within <1 s; the rate constant is of the order of 10^6 M^{-1} s^{-1} as determined by stopped-flow measurement.[3] The spectrum of authentic ebselen Se-oxide (**3**, 50 μM) under the same conditions was not changed upon addition of peroxynitrite (60 μM) (data not shown).

Difference spectra of the reaction mixtures were almost identical to that of authentic ebselen Se-oxide (**3**) (Figure 1B). The relative absorbance change at 279 nm against that at 327 nm, ΔA (279 − 327 nm), resulting from the reaction with peroxynitrite (0−25 μM) was in proportion to the peroxynitrite concentration (Figure 2) and was completed at excess peroxynitrite over the 25 μM ebselen (**2**) present. This indicates a stoichiometry of 1:1.

Identification of Reaction Products. Peroxynitrite (1.1 mM) was added dropwise to a solution of ebselen (**2**, 0.5 mM) in 0.1 M sodium phosphate buffer (pH 10.0)/ethanol = 7:3 (200 μL) and left standing at room temperature. For this preparative reaction, ethanol was included to obtain a higher concentration of **2**. TLC analysis and the comparison with authentic samples showed nearly complete consumption of **2** and production of its Se-oxide (**3**) as the sole product. HPLC analysis and the coinjection with authentic samples showed nearly complete consumption of **2** and production of **3** as well. The HPLC analysis was done with an eluent of 0.1 M aqueous ammonium acetate/acetonitrile, for which the gradient was from 80/20 to 5/95 over 15 min at a flow rate of 1.0 mL/min. Ebselen Se-oxide (**3**) appeared as twin peaks which correspond to **3** and its hydrolyzed form, **3a**.

Yield of Ebselen Se-Oxide and Stoichiometry. For further analysis of the peroxynitrite-mediated oxidation of ebselen (**2**), 10 mM sodium phosphate buffer (pH 7.4)/acetonitrile was chosen as an eluent (details, see Materials and Methods), and good separation was obtained (Figure 3). The yield of ebselen Se-oxide (**3**) by peroxynitrite-mediated oxidation of **2** was observed under various peroxynitrite concentrations. As shown in Figure 4, **2** was almost quantitatively converted to its Se-oxide (**3**), and the yield was in proportion to the peroxynitrite concentration as long as **2** was available. Spontaneous oxidation of ebselen (measured as 1.4 ± 0.4 μM) appears as a small nonzero intercept. A stoichiometry of 1:1 was determined from the slope of the plot of product yield (Figure 4). Complete oxidation of **2** was observed with excess peroxynitrite addition.

The relationship between the product yield and the stability of peroxynitrite in the reaction mixture was investigated. For this purpose, **2** was added to buffer solutions of peroxynitrite at various times after initial

[3] H. Masumoto, R. Kissner, W. H. Koppenol, and H. Sies, unpublished work.

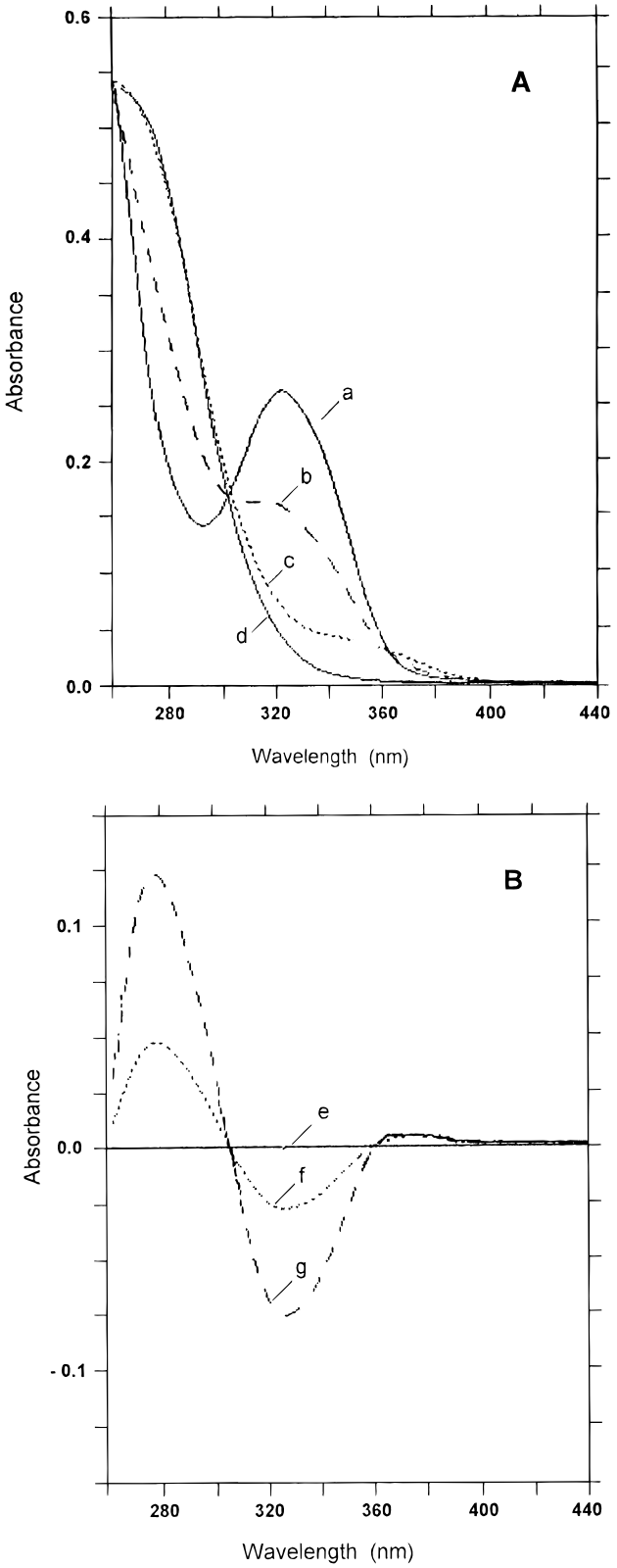


Figure 1. Absorption spectra from the reaction of ebselen with peroxynitrite. (A) Ebselen (**2**, 50 μM) was reacted with peroxynitrite in 0.05 M sodium phosphate buffer (pH 10.9) at room temperature (22 °C). Spectra were recorded immediately before (*a*) and after peroxynitrite addition (30 μM, *b*; 60 μM, *c*). Spectrum of authentic ebselen Se-oxide (**3**, 50 μM) is shown as *d*. (B) Difference spectra were recorded immediately before (*e*) and after peroxynitrite addition (10 μM, *f*; 25 μM, *g*) to a solution of ebselen (**2**, 25 μM).

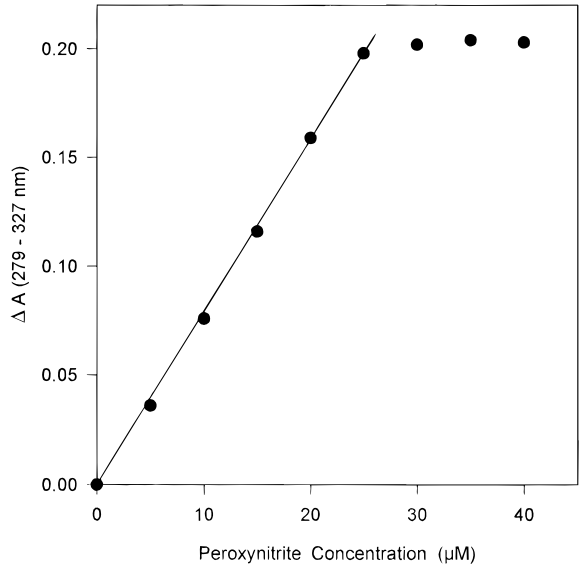


Figure 2. Relationship between ΔA (279 − 327 nm) and peroxynitrite concentration added. Ebselen (**2**, 25 μM) was reacted with peroxynitrite as in Figure 1. Difference spectra were monitored immediately after peroxynitrite addition. Linear least-squares regression line is shown.

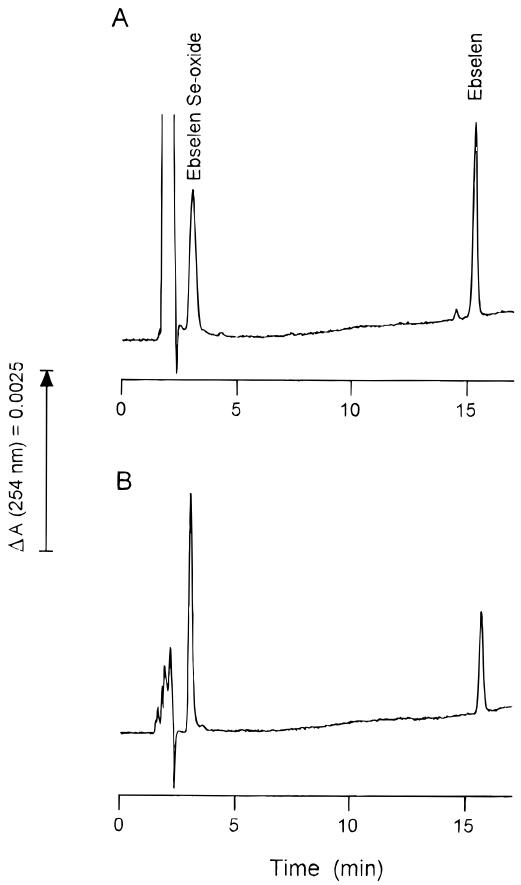


Figure 3. HPLC chromatograms of ebselen and ebselen Se-oxide (A) and reaction mixture samples (B). (A) Authentic ebselen (**2**) and ebselen Se-oxide (**3**) (0.15 and 0.13 nmol, respectively). (B) Ebselen (**2**, 25 μM) reacted with peroxynitrite (20 μM). HPLC condition is as in Materials and Methods.

peroxynitrite addition to start the reaction (for details, see Materials and Methods). The yield of **3** followed an exponential decrease after initial peroxynitrite addition (Figure 5). The slope of the line in Figure 5 corresponds to 1.2×10^{-3} s^{-1}. The value compares well with the rate constant of spontaneous peroxynitrite decay (k_d) in the solution obtained above (ca. 1×10^{-3} s^{-1}). The yield was dependent on the peroxynitrite concentration. The intercept of the extrapolated line with the ordinate, 1.3,

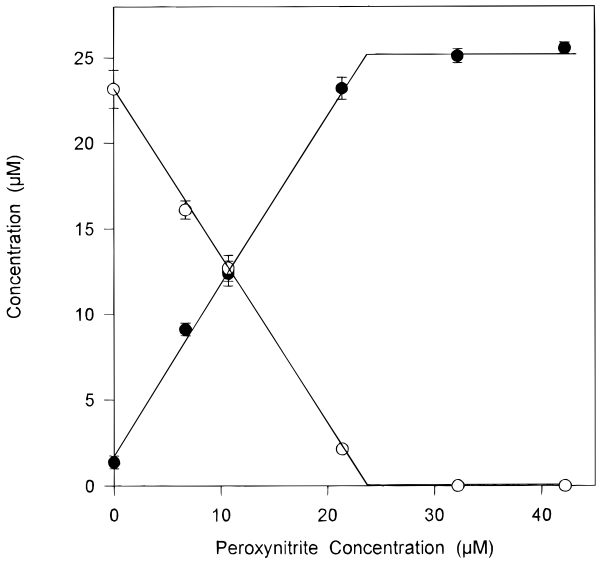


Figure 4. Oxidation of ebselen and formation of ebselen Se-oxide as a function of peroxynitrite concentration. Ebselen (**2**, 25 μM) was oxidized by peroxynitrite in 0.5 M sodium phosphate buffer (pH 10.2) at room temperature (22 °C). Reactions were incubated for 30 min and assayed for the substrate (**2**, ○) and the product (ebselen Se-oxide, **3**, ●) by HPLC. Lines are linear least-squares regression lines. Values are means ± SD (n = 3).

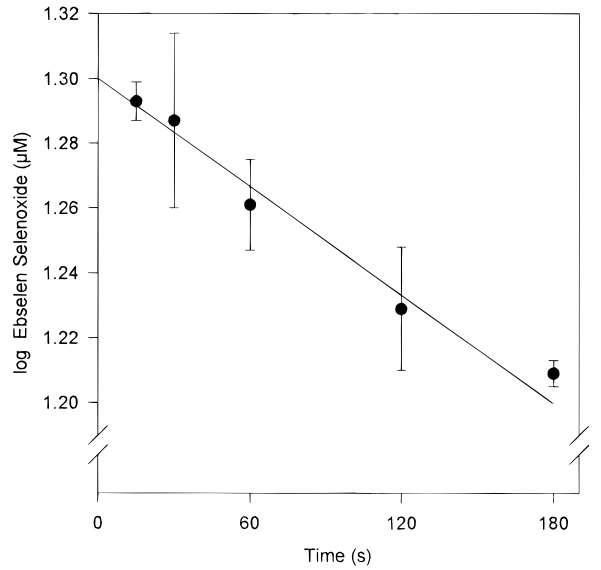


Figure 5. Estimation of theoretical yield of ebselen Se-oxide. Ebselen (**2**, 25 μM) was added to a solution of peroxynitrite (initial 20 μM) as in Figure 4 at the indicated time after initial peroxynitrite addition. Reactions were incubated for 10 min and assayed for the product (ebselen Se-oxide, **3**) by HPLC. Nonlinear least-squares regression line is shown. Values are means ± SD (n = 3).

corresponds to the theoretical yield of **3**, 19.9 μM, in the reaction mixture and is quantitative with respect to the initial concentration of peroxynitrite (20 μM).

Yield of Ebselen Se-Oxide and pH. The reaction occurred also at acidic pH, but was greater at physiological and alkaline pH, where the yield of **3** was almost quantitative (Figure 6). At pH 6.5−10.2, the yield of **3** based on converted **2** was quantitative; i.e., ebselen (**2**) which reacted with peroxynitrite was fully oxidized to **3**. Recovery of **2** plus **3** was less than quantitative at pH 3.6−5.2 (Figure 6).

Effect of Antioxidants. Peroxynitrite-mediated oxidation of ebselen (**2**) was evaluated in the presence of antioxidants at pH 5.3 and 10.2. In this experiment, **2**

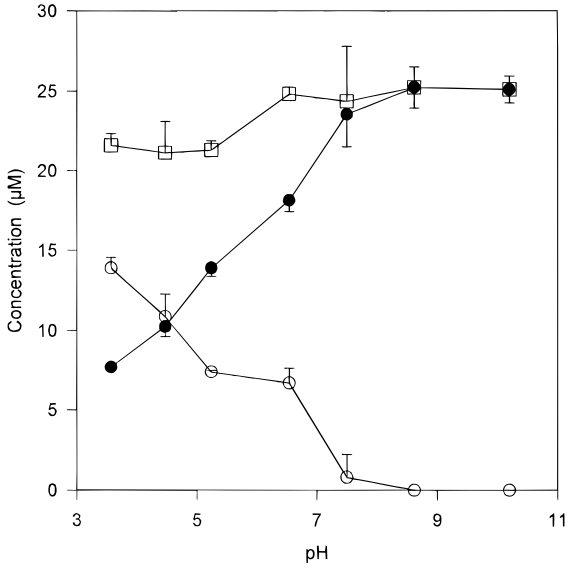


Figure 6. Effect of pH on peroxynitrite-mediated oxidation of ebselen. Ebselen (**2**, 25 μM) was reacted with peroxynitrite (30 μM) in 0.5 M sodium phosphate buffer. Reactions were incubated for 30 min and assayed for the substrate (**2**, ○), the product (ebselen Se-oxide, **3**, ●), and **2** plus **3** (□) by HPLC. The pH values were determined after completion of the reaction. Values are means ± SD (n = 3).

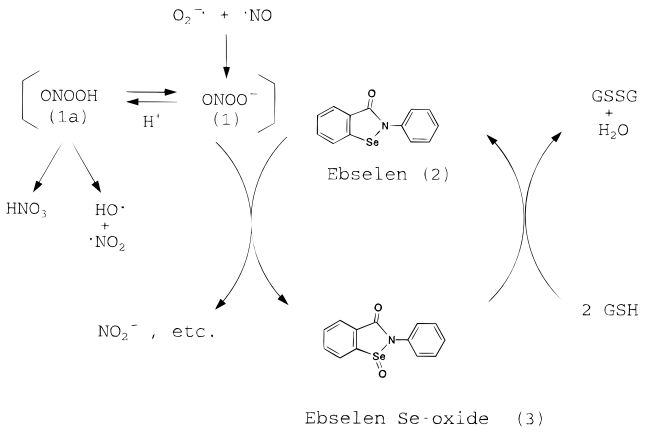
(9.0 and 18.5 μM at pH 5.3 and 10.2, respectively) was dissolved in sodium phosphate buffer without organic solvent as a vehicle and reacted with peroxynitrite (30 μM) at room temperature for 15 min. Methanol (123 mM), DMSO (70 mM), and ethanol (103 mM) did not affect the yield of **3**. Likewise, the radical scavenger mannitol (100 mM) had no effect on the yield: relative yield, 105 ± 6% and 93 ± 5% of control at pH 5.3 and 10.2, respectively. The metal chelator DTPA (100 μM) also had no effect on the yield: relative yield, 102 ± 4% and 95 ± 9% of control at pH 5.3 and 10.2, respectively.

Nitrite Production. A peroxynitrite solution synthesized from hydroxylamine was used for this experiment since this preparation contains low nitrite contamination as byproduct (*27*) as compared to the preparation with the quenched-flow reactor. In order to distinguish nitrite derived from spontaneous decomposition of peroxynitrite and/or nitrite initially present in the reaction mixture from nitrite produced during the reaction with ebselen (**2**), various concentrations of ebselen (0−40 μM) were reacted with a fixed concentration of peroxynitrite (58 μM) in 0.5 M sodium phosphate buffer (pH 10.2) and were quantitatively oxidized. At this peroxynitrite concentration, nitrite contamination in the reaction mixture (background level) was 60 μM. Nitrite production in the reaction with **2** was estimated as the increase of nitrite concentration over the background level. The nitrite production was in proportion to the substrate concentration. A yield of 0.41 mol of nitrite/mol of **2** added was determined from the slope in the plot of nitrite production versus ebselen concentration (data not shown).

Discussion

The results obtained here demonstrate that ebselen (**2**) is oxidized by peroxynitrite rapidly and quantitatively, forming its Se-oxide (**3**) as the sole selenium-containing product at alkaline pH (Figures 3 and 4; Scheme 1). The yield of **3** is governed by the amount of peroxynitrite present in the reaction medium (Figure 5). At physi-

Scheme 1. Potential Detoxication Mechanism of Ebselen against Peroxynitrite[a]



[a] The peroxynitrite-dependent oxidation of ebselen (**2**) to ebselen Se-oxide (**3**) is shown at left. The reduction of ebselen Se-oxide (**3**) to **2** as mediated by GSH, for example, is shown at right.

ological pH, peroxynitrite (**1**) becomes protonated to peroxynitrous acid (ONOOH, **1a**), and **1a** decays rapidly with the first-order rate constant, 1.3 s^{-1}, at 25 °C (*13*). Ebselen (**2**) reacts with peroxynitrite (**1**/**1a**) almost quantitatively at pH 7.5 (Figure 6). The rate constant of the order 10^6 M^{-1} s^{-1} is one of the fastest known for peroxynitrite.[3] Even at acidic pH, where **1a** is predominant, **2** reacts significantly with peroxynitrite (**1**/**1a**), giving **3** as a main product. For example, **3** was obtained as 56% yield at pH 5.2 (Figure 6). In view of the pK_a of 6.8 for **1a**, the lower yield of **3** seems to be due to the reaction rate that is competitive both with the rate of the spontaneous decay process of **1a** at acidic pH and with the rate of pH-dependent formation of byproduct(s) from **2**. The evidence suggests that the reaction between **2** and **1**/**1a** is substantially faster than the spontaneous decay process of **1**/**1a** at physiological pH, even when the concentration of ebselen is in the micromolar range. In addition, radical scavengers have no effect on the yield at acidic as well as alkaline pH. Ebselen (**2**) seems to react with peroxynitrite (**1**/**1a**) directly to give **3**. Both the anion (**1**) and the free acid (**1a**, ground state and energized forms) are likely to be reactive species toward ebselen. Further kinetic study will be needed to specify which is the reactive species.

The main nitrogen-containing product derived from peroxynitrite in the reaction is expected to be nitrite. Nitrite is produced in proportion to the amount of peroxynitrite reacted with **2**, and 40% of peroxynitrite reacted with **2** was recovered as nitrite. The recovery was lower than expected. Nitrate is a possible further product.

Ebselen Se-oxide (**3**) is readily reduced by reducing equivalents such as GSH to revert to **2** (*28*, *31*), constituting an interesting potential defense line against peroxynitrite (Scheme 1). Other reduced species derived from **2** such as selenosulfides (*S*-[2-(phenylcarbamoyl)-phenyl]- derivatives) and selenol (2-(phenylcarbamoyl)-phenylselenol) can be generated in the presence of excess thiols (*32*−*34*). In particular, the selenol is highly reactive with oxidants (*34*, *35*).

Oxidation reactions of peroxynitrite can involve a two-electron process (nucleophilic attack of substrate) or an one-electron transfer reaction (one-electron oxidation by peroxynitrite). A two-electron process with bimolecular displacement by the selenium could be feasible as in the mechanism of oxidation at the sulfur atom of methionine (*7*, *8*). The mechanism is to be studied by kinetic analysis.

A novel principle of the antiinflammatory action of **2** resides in its high reactivity with peroxynitrite which, importantly, leads to oxidation to **3** that can revert to **2** by reducing equivalents such as GSH. This could potentially establish a sustained defense line against peroxynitrite.

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