Kinetic study of the reaction of ebselen with peroxynitrite

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Abstract The second-order rate constant for the reaction of ebselen with peroxynitrite (ONOO$^-$) is $(2.0 \pm 0.1) \times 10^6$ M^{-1} s^{-1} at pH ≥ 8 and 25°C, 3-4 orders of magnitude higher than the rate constants observed for cysteine, ascorbate, or methionine. The activation energy is relatively low, 12.8 kJ/mol. This is the fastest reaction of peroxynitrite observed so far. It may allow Se-containing compounds to play a novel role in the defense against peroxynitrite, one of the important reactive species generated during inflammatory processes.

Key words: Peroxynitrite; Ebselen; Rate constant; Selenoorganic compound

1. Introduction

Peroxynitrite[1] is formed from the near diffusion-controlled reaction of superoxide with nitrogen monoxide [1,2]. The anion is relatively stable, but the protonated form isomerizes to nitrate at a rate of 1.3 s^{-1} at 25°C [3]. During this process, a reactive intermediate is formed that nitrates and hydroxylates phenolic compounds [4-7], and oxidizes molecules such as DMSO [8] and hydrogen peroxide [9,10]. These reactions are first order in peroxynitrous acid (ONOOH) and zero order in the compound that is modified. The rate constant for the modification reaction is the same as that of the isomerization, 1.3 s^{-1}. Compounds reacting with ONOO$^-$ or ONOOH in a bimolecular fashion are sulfhydryls [11], methionine [12], ascorbate [13], iodide [14], carbon dioxide [15], and tryptophan [16]. None of these small molecules react rapidly with ONOO$^-$ or ONOOH (Table 1).

The increasing evidence for a role of peroxynitrite in biological processes [8,17] has generated interest in a potential defense against this reactive oxygen species. We have recently observed that the selenoorganic compound, ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)-one, rapidly reacts with peroxynitrite, yielding the selenoxide, 2-phenyl-1,2-benzisoselenazol-3(2H)-one 1-oxide, as the sole selenium-containing product at 1:1 stoichiometry [18]. Here, we report the second-order rate constant for the reaction of ebselen with peroxynitrite.

2. Materials and methods

2.1. Reagents

Ebselen was a kind gift from Rhône-Poulenc-Nattermann (Cologne, Germany). KO$_2$ was purchased from Fluka (Buchs, Switzerland), NO from Linde (Unterschleißheim, Germany), other chemicals from Merck (Darmstadt, Germany). Peroxynitrite was synthesized by 30% conversion of a solid KO$_2$/quartz sand mixture with NO gas diluted with argon [19]. The solid was dissolved in 0.01 M aqueous sodium hydroxide, and hydrogen peroxide was eliminated by treatment with MnO$_2$ powder. The solution was filtered through a G4 glass filter funnel to remove sand and MnO$_2$. The concentration of the stock peroxynitrite solution was determined at 302 nm ($\varepsilon_{302nm} = 1670$ M^{-1} cm^{-1}) [20] of an aliquot diluted in 0.01 M aqueous NaOH in a Uvikon 820 spectrophotometer (Zürich, Switzerland).

2.2. Kinetic analysis

The kinetic study was carried out on an Applied Photophysics SX17MV stopped-flow apparatus using Spektrakinetik software (Leatherhead, UK). Experiments except the temperature dependence series were performed at 25°C. Ebselen was dissolved to saturation in 0.1 M phosphate buffer. The solution was separated from undissolved solid with a 0.22 µm Millipore filter and was found to contain 15.5 ± 0.5 µM ebselen at ambient temperature. Its pH was adjusted immediately prior to the experiments with solid sodium hydroxide or concentrated phosphoric acid.

Stopped-flow observations were carried out at 322 nm ($\varepsilon_{322nm(ebselen)} = 4030$ M^{-1} cm^{-1}), where the product, ebselen Se-oxide, does not absorb. Peroxynitrite also absorbs at 322 nm ($\varepsilon_{322nm(ONOO^-)} = 1308$ M^{-1} cm^{-1}). Since both components consume each other at the same rate, both absorbance decreases add to the optical changes recorded.

In order to suppress further oxidation of the selenoxide, ebselen was used in excess over peroxynitrite, which was achieved by asymmetric mixing of 15 µM solutions with a ratio of 10:1. This approach leads to a low amplitude with a low signal-to-noise ratio. 13 kinetic traces were recorded and averaged for each determination.

3. Results

The averaged curves show pseudo first-order behavior with amplitudes from 1.7 to 4.6 milliabsorbance units. The residual noise left is strictly periodic and stems from power supply fluctuations of the xenon light source. Fig. 1A,B shows typical traces when excess ebselen is mixed with peroxynitrite at pH 6.8 and 10.7, respectively. The averaged traces were analyzed by fitting to a single exponential function.

The reaction of ebselen with peroxynitrite is influenced by pH and temperature. The reaction is faster with the anion than with the acid, which is contrary to most known reactions of peroxynitrite (Fig. 2). The rate constant is $(2.0 \pm 0.1) \times 10^6$ M^{-1} s^{-1} at pH ≥ 8, which is the highest measured so far with a small molecule (Table 1).

The rate constant varied from 0.8×10^6 to 3.5×10^6 M^{-1} s^{-1} between 3 and 38°C. The Arrhenius plot yields an activa-
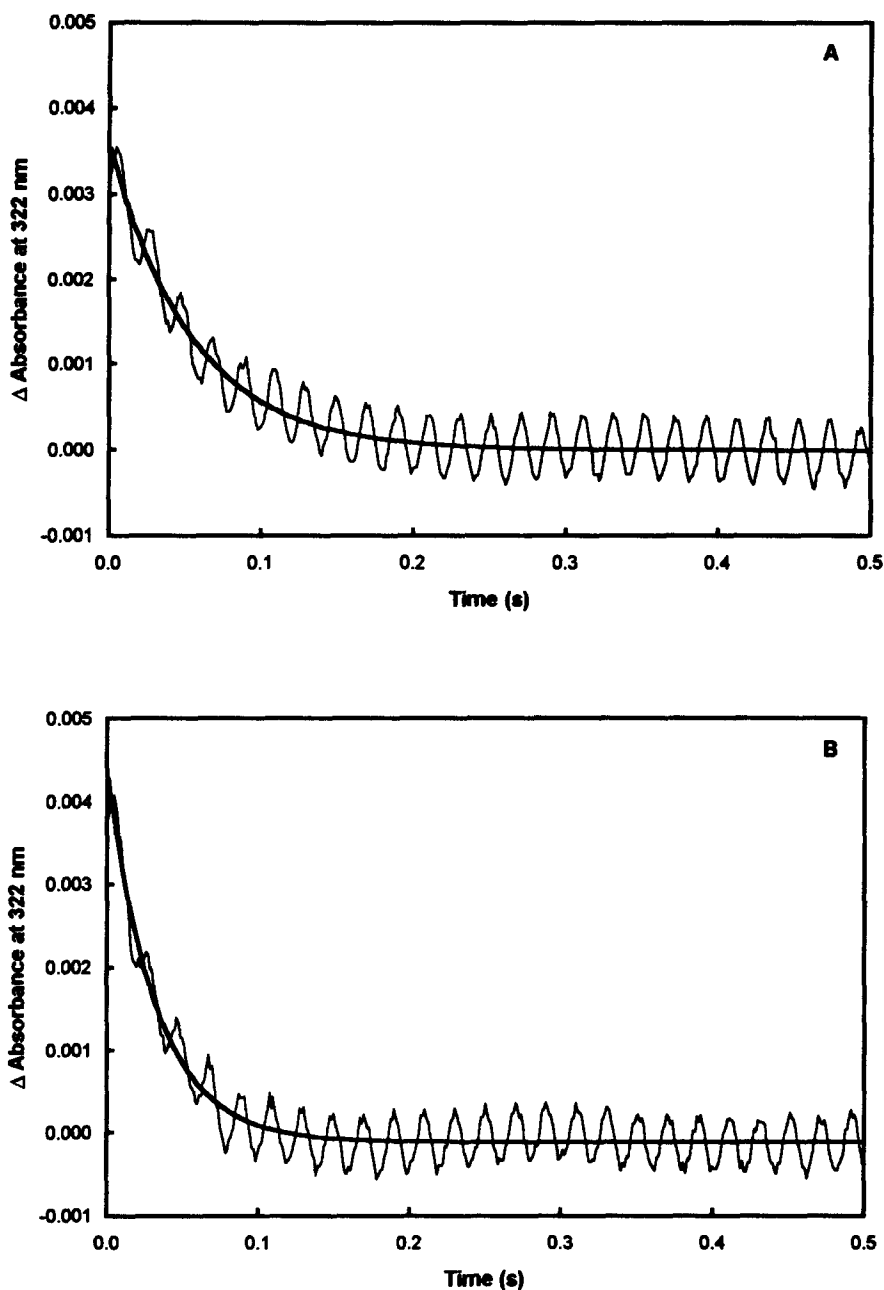
Fig. 1. Stopped-flow detection of ebselen oxidation by peroxynitrite. Ebselen (15.5 μM) in 0.1 M phosphate buffer and ONOO⁻ (15 μM) in 0.01 M aqueous NaOH were mixed (10:1) at 25°C: (A) pH 6.8, (B) pH 10.7. The reaction was followed at 322 nm. The thick solid line is a fit to the reaction of ebselen and ONOO⁻.

Table 1
Second-order rate constants for the reaction with ONOO⁻/ONOOH

Compound	k (M^{-1} s^{-1})	T (°C)	Reference
Ebselen	$(2.0 \pm 0.1) \times 10^6$ (pH ≥ 8)[a]	25	this work
Cysteine	5.9×10^3 [a]	37	[11]
Methionine	9.5×10^2 [b]	25	[12]
Ascorbate	2.4×10^2 [b]	25	[13]
Tryptophan	1.3×10^2 [b]	25	[16]
Iodide	2.3×10^4 [b]	27	[14]
CO$_2$	3×10^4 [a]	24	[15]
Myeloperoxidase	2.0×10^7 [b]	12	[21]
Cytochrome c^{2+}	2.3×10^5 [b]	25	[22]
Alcohol dehydrogenase	$(2.6–5.2) \times 10^5$ (pH 7.4)[c]	23	[23]

[a]Reaction with ONOO⁻.
[b]Reaction with ONOOH.
[c]Reaction with ONOO⁻/ONOOH.

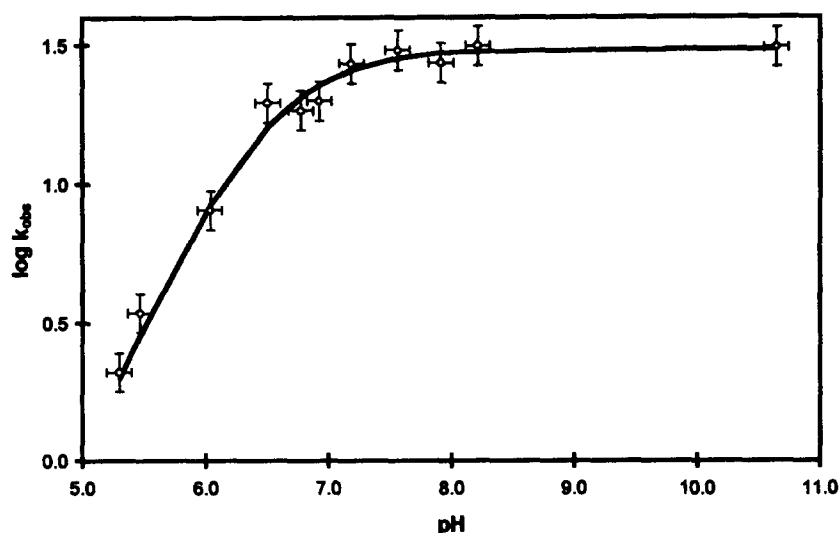Fig. 2. pH profile of the logarithm of the rate constants observed for the reaction of ebselen with ONOO⁻. Conditions as in Fig. 1.

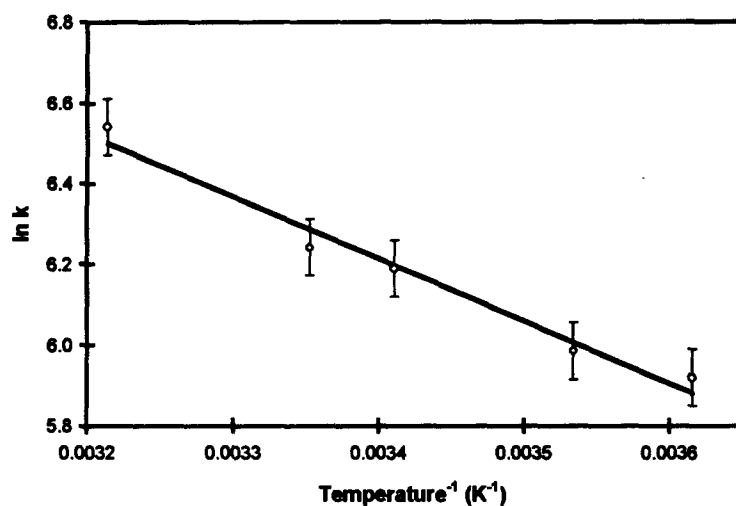Fig. 3. Arrhenius plot for the reaction of ebselen with ONOO⁻. Ebselen (15.5 μM) in 0.1 M phosphate buffer (pH 7.9) and ONOO⁻ (15 μM) in 0.01 M NaOH were mixed (10:1) at 3, 10, 20, 25, and 38°C.

tion energy of 12.8 ± 1.1 kJ/mol and a frequency factor of about 5×10^5 s⁻¹ (Fig. 3). These parameters should be considered to be approximations only because of the limited temperature range.

4. Discussion

Ebselen reacts with peroxynitrite in a bimolecular fashion, giving the selenoxide of the parent molecule [18]. This is confirmed as the observed rate constant depended on the concentration of the major component in preliminary experiments with an excess of peroxynitrite (not shown). There are two remarkable features of this reaction. Firstly, it is the fastest reaction observed so far for a small molecule; only some enzymes and proteins, such as myeloperoxidase [21], cytochrome c [22], and alcohol dehydrogenase [23] react with peroxynitrite substantially faster than small molecules (Table 1). For myeloperoxidase a rate constant of 2.0×10^7 M⁻¹ s⁻¹ at 12°C for the reaction with ONOOH has been calculated [21], an order of magnitude higher than that of ebselen, but it needs to be corrected for multiple target sites on the protein. Secondly,

the dependence on pH is opposite to that of most known redox reactions of peroxynitrite. In the case of ebselen, the anion (ONOO⁻) is the reactive species, which implies that the mechanism must be different from common oxidations with ONOOH. The high reaction rate, the monophasic kinetic trace and the low activation energy suggest that it is a simple oxygen atom transfer (Scheme 1). It seems that the lower rates of reactions of ONOOH referred to in Table 1 are caused by complex mechanisms, whereas reactions of ONOO⁻ consist of a single step.
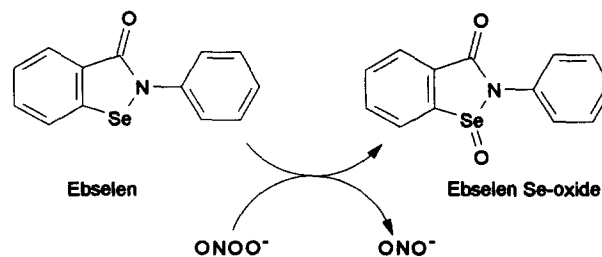
Scheme 1. Peroxynitrite-dependent oxidation of ebselen.

The selenoxide can be converted back to the parent molecule by reduction with thiols [24–26]. The reaction of ebselen may allow Se-containing compounds to play a novel role in the defense against peroxynitrite, an important reactive species in inflammatory processes [17,18,27]. It will be an interesting question whether selenoproteins carry out an analogous function in biology.

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