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Reaction of diphenyl diselenide with hydrogen peroxide and inhibition of delta-aminolevulinate dehydratase from rat liver and cucumber leaves

M. Farina, N.B.V. Barbosa, C.W. Nogueira, V. Folmer, G. Zeni, L.H. Andrade, A.L. Braga and J.B.T. Rocha

Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil

Abstract

The interaction of the product of H_2O_2 and $(PhSe)_2$ with δ-aminolevulinate dehydratase (δ-ALA-D) from mammals and plants was investigated. $(PhSe)_2$ inhibited rat hepatic δ-ALA-D with an IC_{50} of 10 µM but not the enzyme from cucumber leaves. The reaction of $(PhSe)_2$ with H_2O_2 for 1 h increased the inhibitory potency of the original compound and the IC_{50} for animal δ-ALA-D inhibition was decreased from 10 to 2 µM. δ-ALA-D from cucumber leaves was also inhibited by the products of reaction of $(PhSe)_2$ with H_2O_2 with an IC_{50} of 4 µM. The major product of reaction of $(PhSe)_2$ with H_2O_2 was identified as seleninic acid and produced an intermediate with a λ_{max} at 265 nm after reaction with t-BuSH. These results suggest that the interaction of $(PhSe)_2$ with mammal δ-ALA-D requires the presence of cysteinyl residues in close proximity. Two cysteine residues in spatial proximity have been recently described for the mammalian enzyme. Analysis of the primary structure of plant δ-ALA-D did not reveal an analogous site. In contrast to $(PhSe)_2$, seleninic acid, as a result of the higher electrophilic nature of its selenium atom, may react with additional cysteinyl residue(s) in mammalian δ-ALA-D and also with cysteinyl residues from cucumber leaves located at a site distinct from that found at the B and A sites in mammals. Although the interaction of organochalcogens with H_2O_2 may have some antioxidant properties, the formation of seleninic acid as a product of this reaction may increase the toxicity of organic chalcogens such as $(PhSe)_2$.

Correspondence
J.B.T. Rocha
Departamento de Química
Centro de Ciências Naturais e Exatas
Universidade Federal de Santa Maria
97105-900 Santa Maria, RS
Brasil
Fax: +55-52-20-8031
E-mail: jbtrocha@base.ufsm.br

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Key words
- δ-Aminolevulinate dehydratase
- Diphenyl diselenide
- Seleninic acid

Introduction

The molecular mechanisms underlying selenium toxicity are still not thoroughly understood; however, several decades ago Painter (1) emphasized that the toxicity of inorganic selenium could be related to the oxidation of thiols with the concomitant formation of derivatives of the RSSeSR type (selenotrisulfides). After the theoretical Painter proposal, interaction of thiols with inorganic selenium was confirmed experimentally by various investigators (2-5).

Organic selenocompounds such as selenocystine and a variety of diselenides can also react with thiols such as cysteine, dithiothreitol, and reduced glutathione to produce selenocysteine and selenols, respectively, and di-

sulfides (6,7). Although some decades ago the reduction of diselenides to selenols by reaction with thiols was considered to be of physiological significance (6), it is now apparent that the physiological role of selenium involves the participation at the active center of glutathione peroxidase and phospholipid hydrogen glutathione peroxidase (8,9). In these two enzymes the selenium is part of a residue of selenocysteine (10) and during the catalytic cycle of the enzyme the selenol group is probably oxidized by peroxide to a selenenic acid. In subsequent steps, selenol is regenerated by the reaction of selenenic acid with reduced glutathione (8,9). Interestingly, it has been demonstrated that simple diselenides such as diphenyl diselenide (11,12) and the antioxidant selenocompound, ebselen (13-15) accelerate the rate of peroxide reduction by a variety of thiols in a reaction similar to that catalyzed by glutathione peroxidase. However, in addition to taking part in these types of reactions, simple organochalcogens such as diphenyl diselenide catalytically accelerate the rate of thiol oxidation even in the absence of H_2O_2 (16,17). Although the peroxidase-like activity of diselenides may account for their antioxidant properties, the sulfhydryl-disulfide exchange catalyzed by these compounds may contribute to their toxic properties by the oxidation of sulfhydryl proteins.

δ-Aminolevulinate dehydratase (δ-ALA-D) or porphobilinogen synthase is an enzyme sensitive to sulfhydryl-blocking agents (18-22) and participates in the second step of heme, chlorophyll and corrin biosynthesis. This enzyme catalyzes the condensation of two molecules of 5-aminolevulinic acid to porphobilinogen (23), thus playing a fundamental role in animals and in photosynthesizing organisms. Consequently, δ-ALA-D inhibition may impair heme biosynthesis (24) and can result in the accumulation of ALA, which may affect the aerobic metabolism and may have some prooxidant activity (25,26). Although the basic mechanism of catalysis seems to be similar in all phyla (23), the enzyme obtained from mammals and plants exhibits some structural differences (23,27). Recently, we reported that δ-ALA-D from plants, in marked contrast to δ-ALA-D from mammals, was not inhibited by diphenyl diselenide (16). The different sensitivity of the animal and plant enzymes to inhibition by diphenyl diselenide was tentatively associated with differences in the primary structure of δ-ALA-D from these sources (21,23,27).

As stated previously, a variety of organochalcogens exhibit peroxidase-like activity and during the catalytic cycle of H_2O_2 reduction intermediates such as RSeOH and RSeOOH are certainly formed and later reduced by thiols to the original compound (8,9). Although the formation of selenocysteinenic acid at the active site of glutathione peroxidase is presumed to be of physiological significance during H_2O_2 decomposition by reduced glutathione, the reaction of organochalcogens with H_2O_2 *in vivo* may yield more reactive compounds with distinct toxicological properties.

However, to the best of our knowledge, no previous study has investigated the interaction between peroxides and diphenyl diselenide with regard to the reactivity of the products of this reaction towards biomolecules. In an attempt to better understand the toxicology of diphenyl diselenide, a simple organochalcogen compound that plays an important role as an electrophilic reagent for the synthesis of a variety of potential pharmacologically active compounds (28,29), we examined the interaction of this compound with H_2O_2 and that of the product, seleninic acid, with δ-ALA-D from mammals and plants.

Material and Methods

Tissue preparation

Adult rats from our own breeding colony were maintained in an air conditioned room (20-25ºC) under natural lighting conditions,

with water and food (Guabi, Ribeirão Preto, SP, Brazil) *ad libitum*. Animals were anesthetized with ether and killed by decapitation. The livers were quickly removed, placed on ice and homogenized in 7 volumes of 150 mM NaCl. The homogenate was centrifuged at 4,000 *g* at 4ºC for 10 min to yield a low-speed supernatant fraction (S1) that was used for the enzyme assay. Cucumber seeds were germinated for 5 to 7 days at 25ºC. Leaves were homogenized in 5 volumes of medium containing 10 mM Tris-HCl, pH 9.0. The homogenate was then centrifuged as described for animal tissue, and the low-speed supernatant (S1) obtained was used for the enzyme assay. Blood δ-ALA-D from pig liver was partially purified as described by Emanuelli et al. (30).

Reaction of PhSeSePh with hydroperoxide

To study the effect of the products of 4 nmol to 1.2 µmol of PhSeSePh with H_2O_2 (125 µmol), the reaction was carried out for 1 h at 39ºC. Then, catalase (200 units) was added to eliminate the remaining H_2O_2 and the enzymatic reaction was carried out as described above.

To characterize the product of the reaction described above, 100 µmol of PhSeSePh was allowed to react with 4.4 mmol of H_2O_2 at 39ºC. After 4 h, the organic phase was extracted into ethyl acetate and, after solvent evaporation, the melting point of the resulting solid was obtained (121-122ºC). The resulting compound, tentatively identified as benzeneseleninic acid ($PhSeO_2H$), was dissolved in 60% dioxane buffered with phosphate buffer, pH 6.0, and reacted with t-BuSH at a ratio of 1:4 (mol/mol SH) as described by Kice and Lee (29). The formation of the product was also confirmed by analysis of the ^1H- and ^{13}C-NMR spectra.

Enzyme assay

Rat δ-ALA-D activity was assayed according to the method of Sassa (31) by measuring the rate of product (porphobilinogen) formation except that 84 mM potassium phosphate buffer, pH 6.4, and 2.5 mM δ-ALA were used. For the plant enzyme, the medium contained 100 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$ and 3.6 mM ALA. The products of reaction of PhSeSePh and hydroperoxide or PhSeSePh alone were preincubated for 10 min with the enzyme preparations and then the δ-ALA-D reaction was initiated by adding substrate. Incubations were carried out for 1 h at 39ºC for the animal enzyme and at 35ºC for the plant enzyme. The enzyme reaction product was determined using modified Ehrlich's reagent at 555 nm, with a molar absorption coefficient of 6.1×10^4 M^{-1} for the Ehrlich-porphobilinogen salt.

Protein quantification

Protein was measured by the method of Bradford (32) using bovine serum albumin as standard.

IC_{50} determination

The IC_{50} for *in vitro* inhibition of liver and plant δ-ALA-D activity was calculated by the method of Dixon and Webb (33).

Statistical analysis

Data were analyzed by two- or four-way ANOVA as indicated in legend to the figures. Significant main effects or low level interactions were only considered when higher order interactions were not statistically significant.

Results

In the presence of liver supernatant, addition of H_2O_2 did not change the effect of PhSeSePh on δ-ALA-D (Figure 1). At the concentrations studied, H_2O_2 did not affect δ-ALA-D, possibly due to the presence of

catalase in the liver preparation. The presence of catalase under the conditions of the δ-ALA-D assay was determined by titrating H_2O_2 with $KMnO_4$. The concentration of H_2O_2 was reduced to 98-100% of the initial concentration during the 10-min preincubation (data not shown).

In order to circumvent the degradation of H_2O_2 by catalase, PhSeSePh was reacted with H_2O_2 before the addition of liver or plant preparations. Excess H_2O_2 was degraded by adding purified catalase. ANOVA revealed a significant main effect of PhSeSePh concentration, pre-reaction with H_2O_2 and source of δ-ALA-D (all $P<0.01$). Second and third order interactions were also significant (all $P<0.03$). As illustrated in Figure 2A, PhSeSePh inhibited rat liver δ-ALA-D, although the δ-ALA-D from cucumber leaves was inhibited only when PhSeSePh was pretreated with H_2O_2 (Figure 2B). The IC_{50} for plant δ-ALA-D after reaction of PhSeSePh with H_2O_2 was 4 µM. The IC_{50} for liver inhibition by PhSeSePh was 10 µM and the value decreased to 2 µM after reaction of H_2O_2 with PhSeSePh before addition of the enzyme. Similar results were obtained with partially purified δ-ALA-D from pig blood (data not shown). Dithiothreitol, a reducing and sulfhydryl-protecting agent, at 2 mM blocked the effect of PhSeSePh or the products of the reaction of PhSeSePh with H_2O_2. This occurred in every case in which inhibition of δ-ALA-D was detected (Figure 2). The compound synthesized for UV and ^{1}H-NMR spectroscopic characterization was also tested as a δ-ALA-D inhibitor. The inhibitory potency of the synthesized compound was similar to that of the compound(s) generated *in situ* in the mammalian and cucumber δ-ALA-D assay (data not shown).

Reaction of PhSeSePh with excess H_2O_2 can result in the formation of selenenic and seleninic derivatives of PhSeSePh (29,34,35). The major product of reaction between

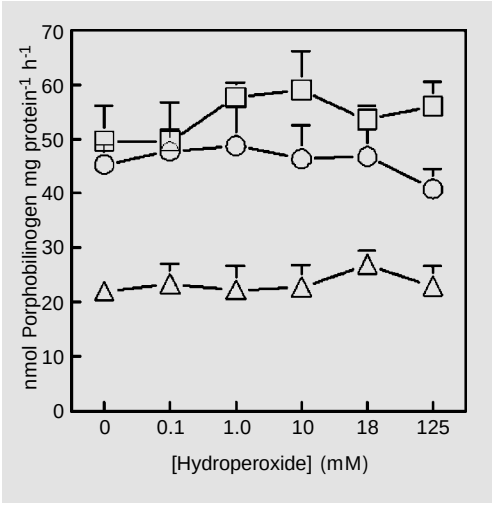


Figure 1. Effect of H_2O_2 and PhSeSePh on rat liver δ-ALA-D. H_2O_2 was added at the same time as the liver supernatant to medium containing 84 mM sodium phosphate buffer, pH 6.4. After 10 min of preincubation, the δ-ALA-D reaction was started by adding ALA to a final concentration of 2.5 mM (control, circles), 0.5 µM PhSeSePh (squares), and 4 µM PhSeSePh (triangles). Two-way ANOVA yielded a significant main effect of PhSeSePh concentration [$F(2,54) = 12.3$, $P<0.01$]. The main effects of H_2O_2 and PhSeSePh x H_2O_2 interaction were not significant.

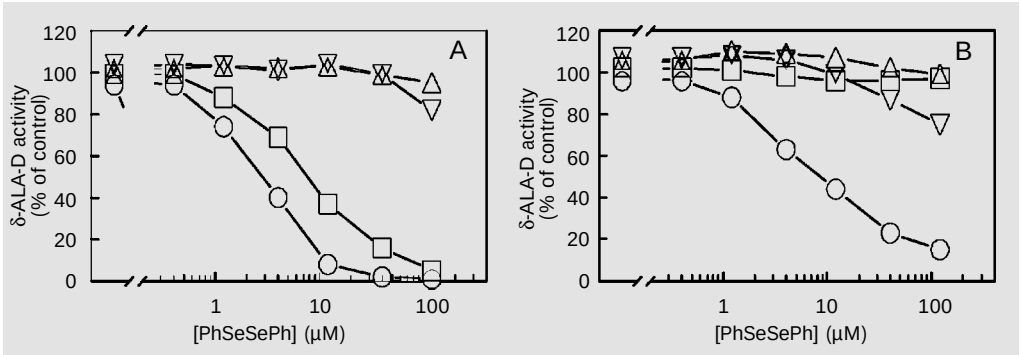


Figure 2. Effect of PhSeSePh on rat liver (A) and cucumber leaf (B) δ-ALA-D. Reaction of PhSeSePh with H_2O_2 was carried out for 1 h and the remaining H_2O_2 was eliminated by adding catalase (200 units). Preincubation of enzyme with PhSeSePh or products from reaction of PhSeSePh and H_2O_2 was initiated by adding liver (A) or plant (B) supernatants to reaction mixtures containing 84 mM phosphate buffer, pH 6.4 (liver), or 50 mM Tris buffer, pH 9.0 (plant). The δ-ALA-D reaction was started 10 min later by adding ALA (final concentration 2.5 or 3.6 mM, respectively). Control (circles); 125 mM H_2O_2 (squares); 2 mM dithiothreitol (DTT, triangles); 125 mM H_2O_2 and 2 mM DTT (inverted triangles). Four-way ANOVA yielded a variety of significant main and interaction effects, which, however, are not presented because the fourth order interaction (7 PhSeSePh concentration x 2 reaction with H_2O_2 x 2 δ-ALA-D source x 2 DTT concentration) was significant [$F(6,168) = 32.3$, $P<0.01$]. This indicates that the effect of PhSeSePh varied depending on the source of the enzyme, on the previous reaction with H_2O_2, and on the presence or absence of DTT.

PhSeSePh and H_2O_2 was isolated and showed a melting point of 120-121ºC, in agreement with published values for benzeneseleninic acid (29,34). The product was then reacted with t-BuSH as described by Kice and Lee (29). This generated an intermediate having a λ_{max} at 265 nm (Figure 3, curve B) and was first assumed to be the thiolseleninate PhSe(O)SR. After 1 h (Figure 3, curve C), the concentration of this intermediate decreased. This was expected because after its initial formation thiolseleninate reacts with the excess of t-BuSH to initiate a reaction sequence leading to PhSeSR and RSSR (29). The formation of benzeneseleninic acid as the major product of reaction of H_2O_2 and diphenyl diselenide was confirmed by analysis of the ^{1}H-NMR spectrum. In fact, the ^{1}H-NMR spectrum of the diphenyl diselenide gives rise to a multiplet at 7.1-7.5 δ in the aromatic region (Figure 4A). In contrast, the ^{1}H-NMR spectrum of the benzeneseleninic acid gives rise to a multiplet at 7.4-7.8 δ in the aromatic region (Figure 4B). Taken together, these data lead us to conclude that the major product of PhSeSePh with H_2O_2 is benzeneseleninic acid. Additional support for the formation of seleninic acid after reaction of PhSeSePh with H_2O_2 was obtained by ^{13}C-NMR spectroscopy. The ^{13}C-NMR spectrum of the diphenyl diselenide showed chemical shifts of carbons between 121 and 130 δ and the product of reaction between PhSeSePh and H_2O_2 showed chemical shifts between 127 and 131 δ. This difference could be explained by the effect of the seleninic group present in this structure and the absence of this group in the structure of diphenyl diselenide.

Discussion

PhSeSePh and benzeneseleninic acid ($PhSeO_2H$) inhibited δ-ALA-D by oxidizing cysteinyl residues essential for enzyme activity, since dithiothreitol abolished the inhibition caused by these compounds. However, the reactivity of these compounds varied depending on the enzyme source. PhSeSePh inhibited only the mammalian enzyme, while $PhSeO_2H$ inhibited δ-ALA-D from all sources. Furthermore, the potency of benzeneseleninic acid was higher on the rat enzyme than that of PhSeSePh, which is consistent with the moderately strong oxidant properties of aromatic seleninic acids (29). Consequently, it is possible that, as a result of the higher electrophilicity of the Se atom in $PhSeO_2H$, the compound reacted more rapidly and/or with less reactive cysteinyl residues in mammalian and plant δ-ALA-D.

The divergent response of the plant enzyme to diphenyl diselenide when compared to mammalian δ-ALA-D is presumably related to differences in the primary structure of these enzymes, especially in the ion-binding regions. The mammalian enzyme possesses two distinct classes of Zn^{2+}-binding sites, the class required for catalytic activity or the A site, that possesses one cysteinyl residue (23,36), and the other Zn^{2+}-binding site, that possesses four cysteine residues

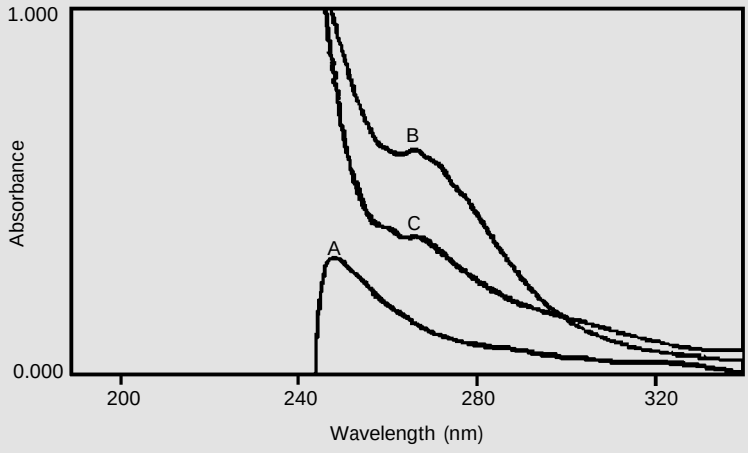


Figure 3. Characterization of the reaction of 2-methyl-2-propanethiol (t-BuSH) with the product of reaction of PhSeSePh with H_2O_2. Benzeneseleninic acid reacted with t-BuSH in 60% dioxane and 25 mM potassium phosphate buffer, pH 6.0. Curves: *A*, 20 µmol t-BuSH; *B*, 5 µmol $PhSeO_2H$ + 20 µmol t-BuSH, immediately after thiol addition; *C*, 5 µmol $PhSeO_2H$ + 20 µmol t-BuSH after 60 min. For curve A the reference cuvette contained 60% dioxane and phosphate buffer, while in curves B and C the reference cuvettes contained the same concentration of $PhSeO_2H$ as the sample cuvette.

(37). In the plant enzyme, no cysteine residues were found in the homologous regions implicated in Zn^{2+} binding in mammalian δ-ALA-D. However, δ-ALA-D from different plant species has approximately four cysteine residues and is inhibited by classical sulfhydryl reagents such as iodoacetamide and N-ethylmaleimide (27). In the present

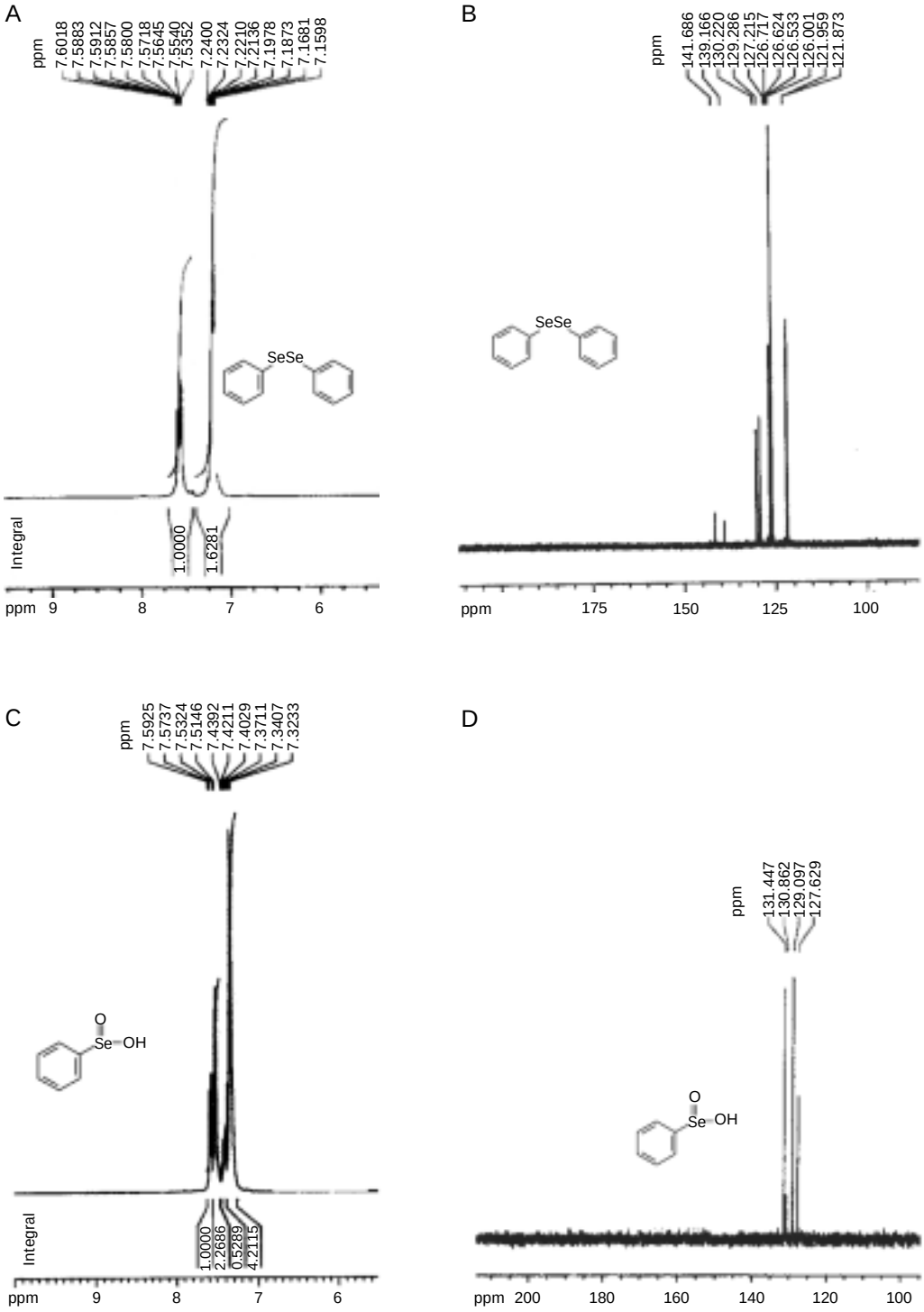
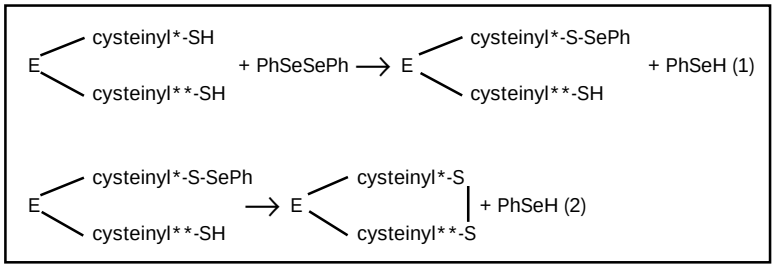


Figure 4. ^{1}H- and ^{13}C-NMR spectra of PhSeSePh and of the product of reaction of PhSeSePh with H_2O_2. The ^{1}H-NMR spectra of PhSeSePh and of the product of reaction of PhSeSePh with H_2O_2 are presented in *A* and *C*, respectively. The ^{13}C-NMR spectra of PhSeSePh and of the product of reaction of PhSeSePh with H_2O_2 are presented in *B* and *D*. Compounds were dissolved in $CDCl_3/D_2O$ and then analyzed with a Bruker DPX-400 (400 MHz) spectrometer.

study, PhSeSePh inhibited mammalian δ-ALA-D with a relatively high potency but had a negligible effect on the plant enzyme. Tentatively, these differences can be explained by the fact that the mammalian enzyme possesses two cysteine residues in spatial proximity at the active site of the enzyme (21), while the plant enzyme does not possess such analogous residues. In view of this, we propose a scheme to interpret the oxidation of mammalian δ-ALA-D by PhSeSePh (see below):

The first step involves the formation of an unstable intermediary of the E-Cys-S-SePh type and PhSeH. Then, in a subsequent step, a less reactive cysteinyl residue (represented as Cys**-SH in the scheme) due to its close proximity to the more reactive residue attacks the sulfur atom of the E-Cys*-S-SePh, producing the oxidized enzyme and a second molecule of PhSeH. Support for this scheme has also been obtained using low molecular weight thiol-containing substances and dithiothreitol (a dithiol) was found to be a better substrate than cysteine or glutathione (monothiols) for the oxidation catalyzed by organodiselenides (7,38). The PhSeH formed after the reaction with thiols is oxidized back to PhSeSePh by atmospheric O_2. In fact, the regeneration of PhSeSePh after reaction with O_2 helps to explain the previous observation that the inhibitory potency of PhSeSePh towards rat δ-ALA-D decreases considerably in an anaerobic atmosphere (16). The inhibitory effect of $PhSeO_2H$ on mammalian δ-ALA-D can be mediated by a direct oxidation of Cys*-SH and Cys**-SH. Oxidation of other less reactive cysteinyl residues other than those shown in the scheme cannot be ruled out.

In the present study, we showed that the product of PhSeSePh with H_2O_2 is more inhibitory for δ-ALA-D than the parent selenide compound. In analogy to the proposed glutathione peroxidase catalytic cycle (8,9), where the Se atom at the active site of the enzyme goes from the selenol to a still not isolated (39,40) but presumed selenenic form, the peroxidase-like activity of diselenides probably involves the formation of a seleninic intermediary during the reduction of peroxides. Thus, if extrapolated to the *in vivo* situation, it is reasonable to suppose that the toxicological properties of PhSeSePh, in addition to a direct interaction with thiol groups of proteins such as δ-ALA-D, may be related, in part, to its oxidation to seleninic acid after reaction with endogenous peroxides. However, since seleninic acid is an extremely reactive compound, the inhibition of δ-ALA-D will occur only if reaction of PhSeSePh with hydroperoxides takes place in the cytosol in close proximity to the enzyme.



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