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AN ESR STUDY OF NONENZYMATIC REACTIONS OF NITROSO COMPOUNDS WITH BIOLOGICAL REDUCING AGENTS

NAOHIRO TAKAHASHI‡, VOLKER FISCHER, JÖRG SCHREIBER and RONALD P. MASON§

Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, PO Box 12233, Research Triangle Park, NC 27709, USA

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The nonenzymatic reduction of nitrosobenzene (I), 2-nitroso-1-naphthol (II) and 2-nitroso-1-naphthol-4-sulfonic acid (III) with reducing agents such as NADPH, L-cysteine and N-acetyl-L-cysteine led to the formation of the corresponding hydronitroxide radicals, as confirmed with ESR spectroscopy. In addition to these radicals, a novel hydronitroxide radical, which was conjugated with GSH at the 4-position, was observed in the reaction of II or III with GSH. The formation of a hydronitroxide conjugated with GSH still retains the radical structure with its related redox chemistry. In this case, the formation of a GSH conjugate does not lead to the formation of chemically less reactive species.

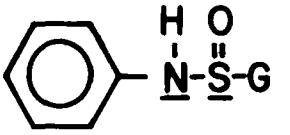
KEY WORDS: ESR, free radicals, nitrosobenzene, glutathione conjugate, GSH

INTRODUCTION

Nitroso and N-hydroxy compounds are considered potential ultimate reactive metabolites of a variety of pharmacologically and toxicologically interesting compounds. It has been reported that these compounds are commonly carcinogenic and/or mutagenic, and also covalently bind to proteins and DNA.[1–5] Arylnitroso derivatives may be formed via the metabolism of virtually all compounds with nitrogen attached to an aromatic system. For example, the dismutation of nitroxide radicals after one-electron oxidation of the corresponding hydroxamic acid forms the nitroso derivative.[6,7]

Oxidation of phenylhydroxylamine in erythrocytes is coupled with the formation of glutathione conjugates, together with the production of methemoglobin from hemoglobin.[8] It has been suggested that nitrosobenzene (I), the two-electron oxidation product of phenylhydroxylamine, is involved in the conjugation with GSH.[8] Eyer[9] has reported that I rapidly reacted with glutathione nonenzymatically to form phenylhydroxylamine, oxidized glutathione and glutathionesulfinanilide.

‡Permanent address: Takarazuka Research Center, Sumitomo Chemical Co. Ltd., 4-2-1 Takatsukasa, Takarazuka, Hyogo 655, Japan.
§Address correspondence to: Ronald P. Mason.



However, by analysis of the equilibrium data, he argued against the production of radical intermediates from I or GSH.

Several nitroso compounds are reduced nonenzymatically with NADPH to form carcinogenic intermediates.[10] Fischer and Mason[11] have reported that nitrosonaphthols such as 1-nitroso-2-naphthol and 2-nitroso-1-naphthol underwent both one-electron oxidation by the peroxidase prototype, horseradish peroxidase, to form iminoxyl free radicals, and nonenzymatic net one-electron reduction with NADPH to yield hydronitroxides.

We now report the identification of nitroxide radicals by ESR spectroscopy during the nonenzymatic reaction of nitrosobenzene (I), 2-nitroso-1-naphthol (II) and 2-nitroso-1-naphthol-4-sulfonic acid (III) with reducing agents such as GSH, L-cysteine and N-acetyl-L-cysteine at physiological pH, and the unexpected formation of GSH conjugates.

MATERIALS AND METHODS

Nitrosobenzene (I), 2-nitroso-1-naphthol (II), NADPH, reduced glutathione (GSH), L-cysteine and N-acetyl-L-cysteine were purchased from Sigma Chemical Company (St. Louis, MO). 2-Nitroso-1-naphthol-4-sulfonic acid (III) was obtained from Pfaltz & Bauer (Stamford, CT). I and II bottled in sealed containers with access through a septum (ISOPAC) were dissolved in ethanol to obtain 83 mM stock solutions. All chemicals were of the highest grade commercially available and used without additional purification.

ESR experiments were performed on a Varian E-109 spectrometer at 9.4 GHz equipped with a TM_{110} cavity and quartz flat cell. All measurements were carried out with 100 kHz field modulation at room temperature. Simulations of ESR spectra were done with a Varian E-935 data acquisition system or on a Nicolet 1180 computer. A computer-aided tuning procedure was used to optimize the hyperfine splitting constants using the correlation between experimental and simulated spectra as the criterion for goodness of fit.[12]

The reaction mixture contained 5 mM of the substrate and 2.5–20 mM of the reducing agent in 0.1 M phosphate buffer (pH 7.4) containing 5 ~ 7% ethanol. The solutions were transferred to the quartz flat cell by means of a rapid sampling device (Gilford Instruments).[13]

RESULTS

Reduction of I to the Hydronitroxide Free Radical

Nonenzymatic reduction of I by reducing agents such as NADPH, GSH, L-cysteine and N-acetyl-L-cysteine was studied in phosphate buffer, pH 7.4, under aerobic

conditions. Figs. 1A and 1B show the ESR spectra obtained from the reduction of I by NADPH and GSH, respectively. Fig. 1B is identical to Fig. 1A, except broader lines were observed with GSH. ESR spectra similar to that in Fig. 1B were also observed during the reduction of I by L-cysteine and N-acetyl-L-cysteine (data not shown). No significant ESR signal was observed in the absence of the reducing agents (Fig. 1C). Fig. 1D shows the computer simulation for Fig. 1A using the hyperfine splitting constants in Table I. The assigned splitting constants were in good accord with those reported for the phenyl nitroxide radical (IV) generated by photolysis of phenylhydroxylamine, nitrobenzene or of I in distilled water[14–16] and by reduction of I with ascorbate.[17]

The formation of the phenyl nitroxide radical (IV) was observed either under air or when nitrogen gas was purged through the incubation solution for 5 min prior to the ESR measurement. In the presence of higher concentrations of the reducing agents (e.g. L-cysteine: 10 mM, NADPH: 5 mM), no ESR signal was observed, possibly indicating the total reduction to the corresponding hydroxylamine.

Reduction of II and III to the Hydronitroxide Free Radicals and Conjugation with GSH

The observed and simulated ESR spectra detected during the reduction of II by NADPH in phosphate buffer at pH 7.4 under aerobic conditions are shown in Figs. 2A and 2B, respectively. The assigned splitting parameters in Table I were in good agreement with those reported for 1-hydroxynaphth-2-yl nitroxide free radical (V).[11] In contrast, the reaction of II with GSH resulted in the ESR spectrum shown in Fig. 2C. The obvious asymmetry indicated the presence of at least two radical species, one of which could be V. A subtraction of Fig. 2B from Fig. 2C gave the spectrum shown in Fig. 2D. The good symmetry of the difference spectrum indicates the quality of the subtraction. The computer simulation for Fig. 2D using the hyperfine splitting constants in Table I is shown in Fig. 2E. The hyperfine splitting constants are very similar for both species (Table I) except that the new species (VI) has lost a single large hyperfine interaction with hydrogen. This new species was assigned to 1-hydroxy-4-glutathionylnaphth-2-yl nitroxide (VI). Reduction of II with L-cysteine or N-acetyl-L-cysteine formed hydronitroxide V, but conditions for conjugate formation could not be found (data not shown).

The incubation of III with NADPH resulted in the radical formation shown in Fig. 3A. The spectrum was simulated as shown in Fig. 3B using the parameters in Table I, indicating the formation of 1-hydroxy-4-sulfonic acidnaphth-2-yl nitroxide (VII),

TABLE I
ESR Parameters Used For Simulations

Radical	Hyperfine splitting constants (Gauss)
Phenyl nitroxide (IV)	N: 10.69, H(NH): 13.27, 2H(*ortho*): 3.59 & 3.32, 2H (*meta*): 1.12, H (*para*): 3.32
1-Hydroxynaphth-2-yl nitroxide (V)	N: 4.32, H: 5.14, 2H: 4.50, 2H: 1.31, H: 0.87, H: 0.41, H: 0.32
1-Hydroxy-4-S-glutathionyl naphth-2-yl nitroxide (VI)	N: 3.84, H: 4.45, H: 4.30, 2H: 1.13, H: 0.98, 2H: 0.52
1-Hydroxy-4-sulfonic acid naphth-2-yl nitroxide (VII)	N: 4.85, 2H: 5.20, H: 1.06, H: 0.90, H: 0.63, H: 0.55, H: 0.45

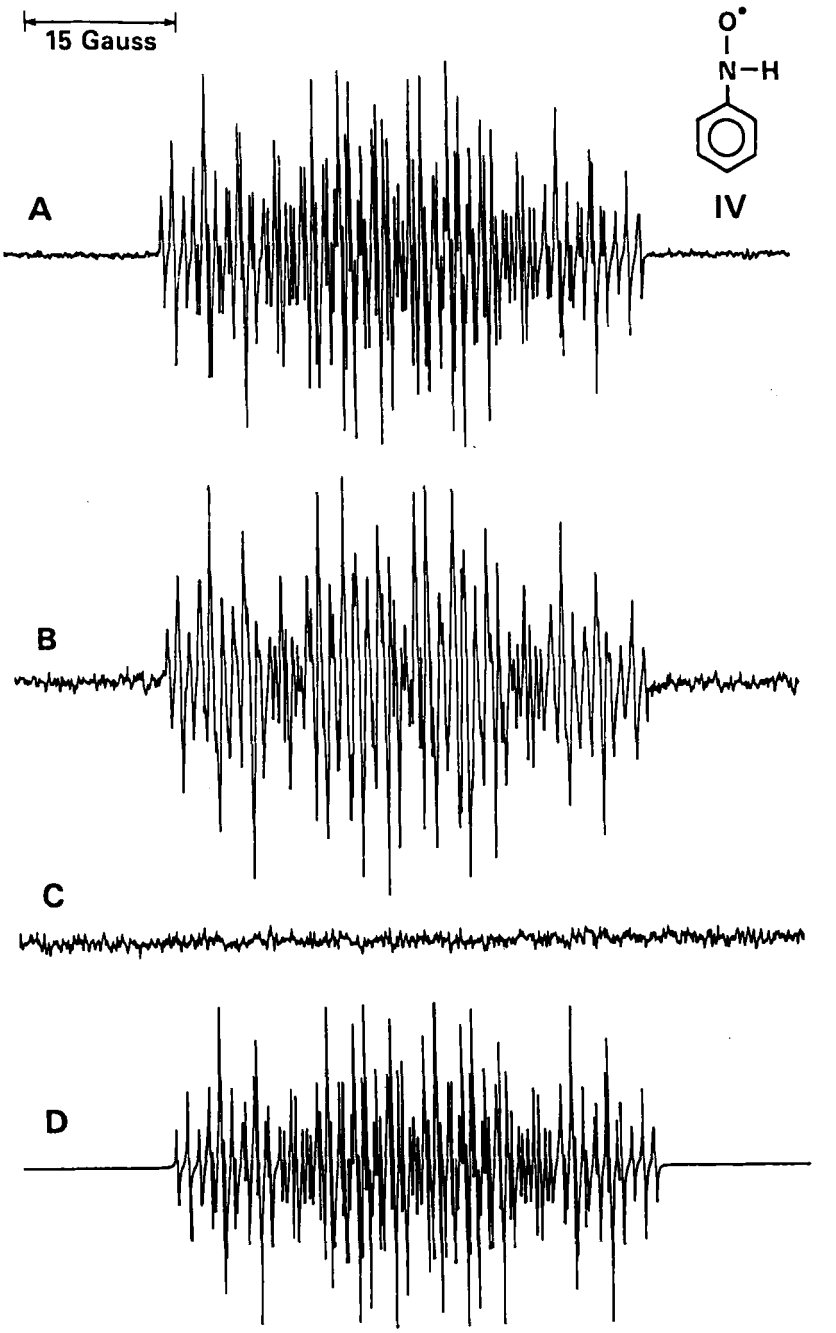


FIGURE 1 ESR spectra of phenyl nitroxide (IV) in phosphate buffer, pH 7.4, under aerobic conditions. (A) 5 mM nitrosobenzene (I) and 2.5 mM NADPH; (B) 5 mM I and 5 mM GSH; (C) same as (B) but in the absence of GSH; (D) computer simulation of (A). Spectrometer setting: 5 mW, microwave power; 85 mG, modulation amplitude; 0.5 sec, time constant; 5 G/min, scan rate.

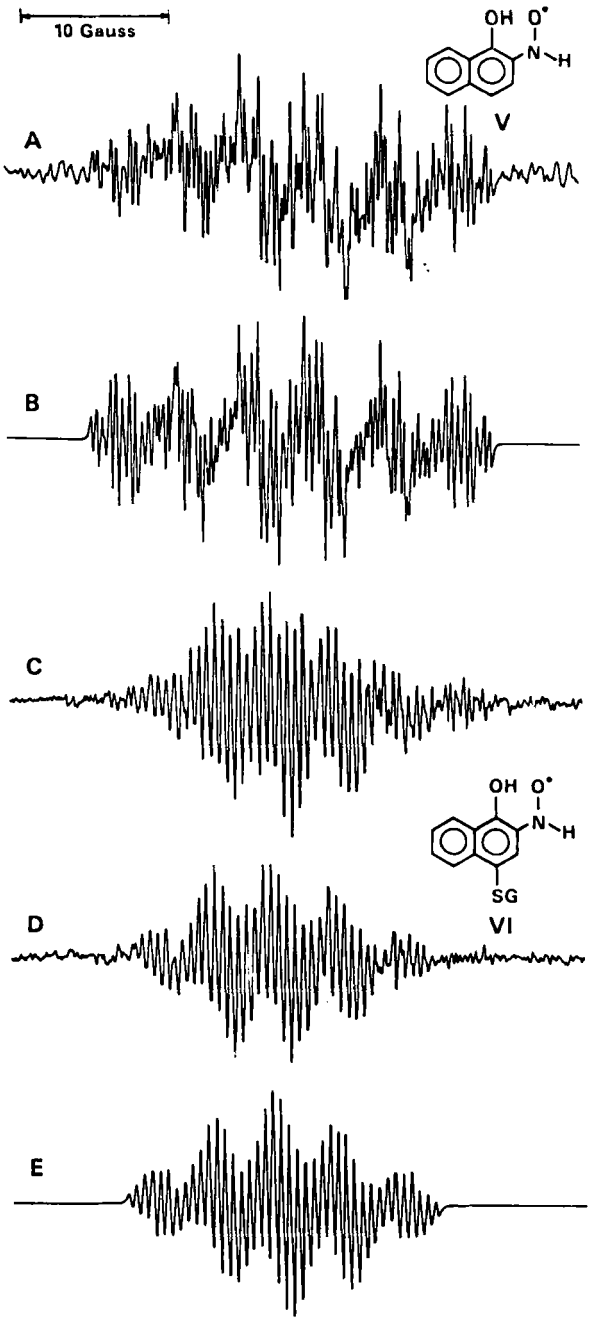


FIGURE 2 ESR spectra obtained by reaction of 2-nitroso-1-naphthol (II) with NADPH or GSH in phosphate buffer, pH 7.4, under aerobic conditions. (A) 5 mM II and 5 mM NADPH; (B) computer simulation of (A); (C) 5 mM II and 25 mM GSH; (D) subtraction of (A) from (C); (E) computer simulation of (D). Spectrometer setting: 2 mW, microwave power; 16.5 mG, modulation amplitude; 2 sec, time constant; 1.3 G/min, scan rate.

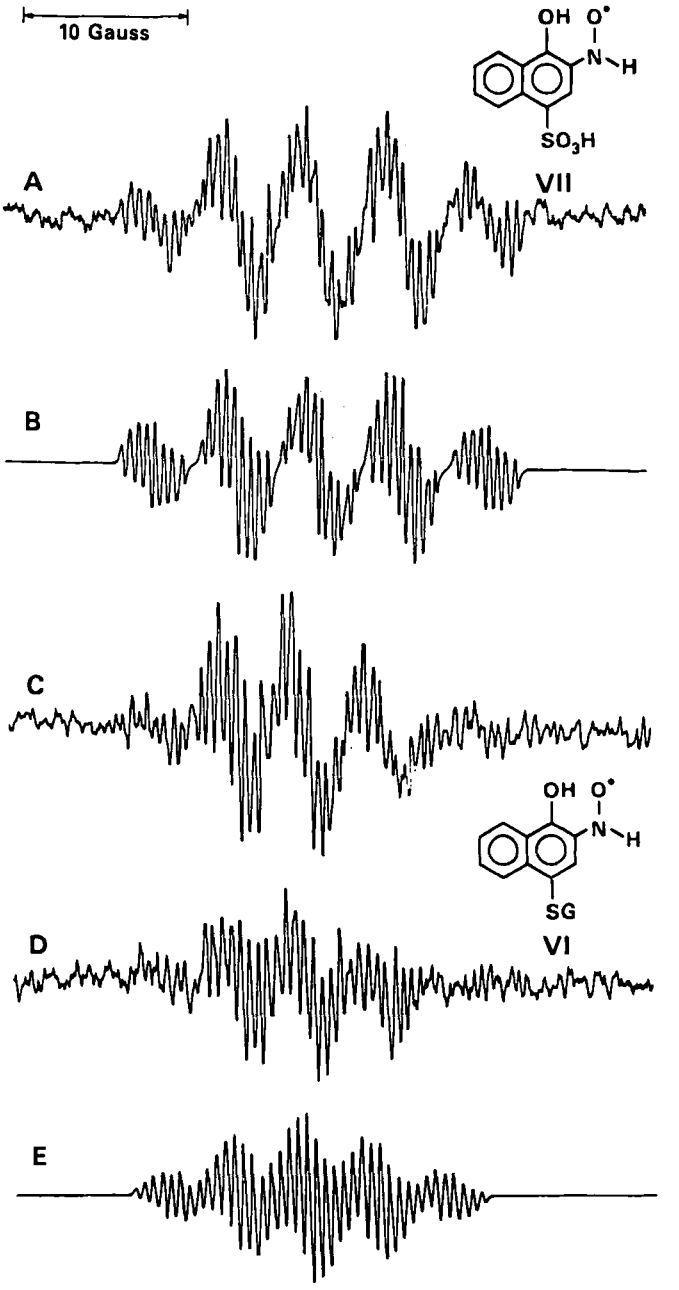


FIGURE 3 ESR spectra obtained by reaction of 2-nitroso-1-naphthol-4-sulfonic acid (III) with NADPH or GSH in phosphate buffer, pH 7.4, under aerobic conditions. (A) 5 mM III and 5 mM NADPH; (B) computer simulation of (A); (C) 5 mM III and 20 mM GSH; (D) the subtraction of (A) from (C); (E) computer simulation of (D). Spectrometer setting: 2 mW, microwave power; 16.5 mG, modulation amplitude; 8 sec, time constant; 0.67 G/min, scan rate.

which again differs from V primarily by the loss of a single large hyperfine interaction with hydrogen. The reaction of III with GSH gave an additional radical species (Fig. 3C). A subtraction of Fig. 3B from Fig. 3C gave the symmetrical spectrum shown in Fig. 3D. This spectrum is apparently due to the same species detected first in Fig. 2D and is assigned to the same nitroxide conjugated with GSH (VI). A simulation for Fig. 3D was carried out using the same parameters as Fig. 2E (Fig. 3E). These spectra indicate the substitution of sulfonate with GSH at the 4-position because of the close similarity of hyperfine coupling constants of both species. The amount of VI formed by the reaction of both II and III with GSH was decreased with lower concentrations of GSH. The centers of the ESR spectra of V and VII were located at a lower magnetic field than that of VI by 0.7 G, consistent with the expected g-shift from a sulfur substitution. Similar results were obtained under a nitrogen atmosphere. Reduction of III with L-cysteine or N-acetyl-L-cysteine formed hydronitroxide VII, but again conditions for conjugate formation could not be found (data not shown).

As in the case of nitrosobenzene, in the presence of higher concentrations of the reducing agents (e.g., NADPH, 10 mM; L-cysteine, 40 mM), no ESR signal was observed, again indicating total reduction to the corresponding hydroxylamines.

DISCUSSION

The reaction of the nitroso compounds, such as I, II and III, with the reducing agents NADPH, L-cysteine and N-acetyl-L-cysteine generated the corresponding hydronitroxide free radicals via a net one-electron reduction. The concentrations of the radicals formed were dependent on the nitroso compounds and the nature of the reducing agents. In the reduction of I, a higher steady-state concentration of the radicals was obtained compared to the reduction of II or III. In addition to IV, V, and VII, the glutathione conjugated radical, VI, was formed in the reaction of II or III with GSH. Eyer has reportedly ruled out the formation of the radical intermediate IV in the nonenzymatic reduction of I by GSH.[9] However, it was demonstrated in the present study that I was nonenzymatically reduced to form the phenyl hydronitroxide radical, IV, via reduction by reducing agents such as GSH, NADPH, L-cysteine and N-acetyl-L-cysteine. Smentowski has reported the reduction of nitrosobenzene to the nitrosobenzene anion radical (the conjugate base of IV) by aromatic thiolates in basic dimethyl sulfoxide-*t*-butyl alcohol.[18] It has been reported that the hydronitroxide radical was also formed by reaction of I with ascorbate[17] or superoxide.[19] The reduction of nitroso compounds to hydronitroxides is not necessarily a one-electron transfer (followed by proton transfer), because the hydronitroxide is also formed by the oxidation of the corresponding hydroxylamine, and the hydroxylamine can be formed directly by a hydride transfer. If the nitroso compound is the oxidizing agent then hydronitroxide formation would be due to a simple comproportionation reaction.[20] Although NADPH presumably reduces nitrosobenzene to phenylhydroxylamine by a hydride transfer, this reaction is unlikely for GSH.

In the case of the reduction of II and III by GSH, the novel hydronitroxide radical (VI) conjugated with GSH at the 4-position was formed in addition to V and VII, although no GSH conjugated radical was observed with nitrosobenzene. This indicates that II underwent net one-electron reduction before or after conjugating with GSH at the reactive 4-position, and that one-electron reduction of III, which contains the sulfonate group, occurred before or after substitution of the sulfonate group with

GSH. In the case of I, II or III, no ESR signal was observed in the presence of excess concentrations of the reducing agents, suggesting total reduction to the corresponding hydroxylamines. Eyer reported that I reacted with GSH to form nonradical products such as glutathionesulfinanilide as well as phenylhydroxylamine.[9] The absence of detectable conjugate formation with L-cysteine and N-acetyl-L-cysteine is probably a reflection of the multi-step reactions required to form these hydronitroxide conjugates, resulting in low concentrations of even the GSH conjugate VI.

It has been shown that several nitrosonaphthols can be metabolized both through oxidative pathways to form iminoxyl free radicals and through reductive pathways to form hydronitroxides.[11] However, a GSH-conjugated hydronitroxide radical such as VI, has never been reported. A conjugate formation with GSH is usually considered to be a detoxification process of toxic chemicals, but it appears that the formation of VI, which is still a radical, is not necessarily a detoxification reaction. We have recently observed that 1,4-naphthosemiquinone and 2-methyl-1,4-naphthosemiquinone react with GSH to form GSH-conjugated radicals such as 2-S-glutathionyl-, 2,3-S,S-diglutathionyl-1,4-naphthosemiquinone and 2-methyl-3-S-glutathionyl-1,4-naphthosemiquinone radicals, respectively.[21] It has been suggested that an aromatic amine, procainamide, was bound to protein sulfhydryl groups following oxidation to a nitroso-metabolite.[22] The free radical intermediates and their GSH conjugates should be considered in examining the reactive species responsible for the toxicity of nitroso compounds.

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