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Glutathione and Ascorbate Reduction of the Acetaminophen Radical Formed by Peroxidase

DETECTION OF THE GLUTATHIONE DISULFIDE RADICAL ANION AND THE ASCORBYL RADICAL*

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The acetaminophen phenoxyl radical was generated by the oxidation of acetaminophen by horseradish peroxidase in a fast-flow ESR experiment, and its reaction with glutathione and ascorbate was studied. Glutathione reduces the phenoxyl radical of acetaminophen to regenerate acetaminophen and form the thiyl radical of glutathione. This thiyl radical reacts with the thiolate anion of glutathione to form the disulfide radical anion, which was detected and characterized by ESR spectroscopy. In the presence of ascorbate, the ascorbyl radical was produced by the reduction of the acetaminophen phenoxyl radical by ascorbate. This reaction results in the complete reduction of the free radical of acetaminophen, whereas the glutathione reduction of the phenoxyl radical of acetaminophen was not complete on the fast-flow ESR time scale of milliseconds. This suggests that ascorbate rather than glutathione is more likely to react with the acetaminophen phenoxyl free radical *in vivo*. In the presence of both ascorbate and higher concentrations of glutathione, the reaction with ascorbate is dominant.

When cysteine was used in the place of reduced glutathione in the above assay system, the disulfide radical anion of cystine was observed in a manner similar to glutathione. These reactions may have significance in the detoxification of acetaminophen and the free radical metabolites of xenobiotics in general. Only in cells containing low levels of ascorbate can glutathione play a direct role in the detoxification of the acetaminophen phenoxyl radical.

During oxidative stress caused by the production of oxygen-derived radicals, glutathione is known to protect the cell by removing hydrogen peroxide in a reaction catalyzed by glutathione peroxidase (1). Glutathione is also known to reduce free radical metabolites of xenobiotics, resulting in the formation of the unchanged parent molecule and the glutathionyl radical and radicals derived therefrom (Scheme 1). This reaction is known as thiol pumping and is suggested as a possible mechanism for the detoxification of the free radical metabolites of xenobiotics in biological systems (2–8). Although Yamazaki and co-workers (2) first reported it in 1969 in ESR studies on the reaction of the chlorpromazine radical cation with glutathione, thiol pumping gained interest only in the past few years (9). The thiyl radical forms the disulfide oxidation product GSSG either by dimerization or through the reaction of oxygen with the disulfide radical anion (Scheme 1).

In this paper, we provide ESR spectroscopic evidence for the formation of the disulfide radical anion of glutathione in a system containing horseradish peroxidase, acetaminophen, and glutathione using the fast-flow ESR technique. We have also demonstrated that ascorbate reduces the phenoxyl radical of acetaminophen completely, whereas glutathione is less efficient in reducing this free radical metabolite of acetaminophen.

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MATERIALS AND METHODS

Acetaminophen, glutathione, horseradish peroxidase type I, horseradish peroxidase type VI (EC 1.11.1.7), hydrogen peroxide, sodium ascorbate, and cysteine hydrochloride were obtained from Sigma.

The ESR fast-flow measurements were carried out using a Varian E-109 spectrometer equipped with a TM_{110} cavity at room temperature. Reagents were prepared in two 4-liter aspirator bottles, one containing glutathione or cysteine and horseradish peroxidase in 0.05 M phosphate buffer and the second bottle containing acetaminophen and hydrogen peroxide in the same buffer. These reagents were prepared by first bubbling the 0.05 M phosphate buffer with nitrogen for 15 min. Then the reagents were added in appropriate quantities, and the pH of the two reagents was adjusted to 7.4. The two bottles, under continuous nitrogen bubbling, were placed at a height of about 2.5 meters and connected to the flow system. The flow system consisted of two Gilmont compact flow meters, the outlets of which were connected with Tygon tubing to a quartz fast-flow mixing chamber flat cell (Wilmad Type WG-804, 17-mm-wide flat cell). The rate of flow of the reagents was regulated by the two flow meters. In most of the experiments the flow rate for each of the reagents was about 60 ml/min. The effluent was delivered to a waste container by connecting Tygon tubing to the outlet of the flat cell. In a typical experiment, glutathione (100 mM) and horseradish peroxidase type I (10 units/ml) in one bottle and acetaminophen (10 mM) and hydrogen peroxide (25 mM) in the second bottle were each flowed at 60 ml/min, and the ESR spectra were continuously recorded. In the experiment using ascorbate, ascorbate was placed in the bottle containing horseradish peroxidase or glutathione and horseradish peroxidase. The experimental spectra were computer-simulated using the reported hyperfine coupling constants. The g values of the disulfide radical anions were measured relative to that of acetaminophen, $g = 2.0043$ (10).

Oxygen consumption by solutions containing various substrates was measured at 30 °C using a Clark oxygen electrode (Yellow Springs Instruments).

RESULTS

Scheme 1 shows the proposed mechanism for the reduction of acetaminophen radical by ascorbate and glutathione. The ESR spectroscopic detection of the disulfide radical anion of cysteine formed in a fast-flow ESR experiment is shown in

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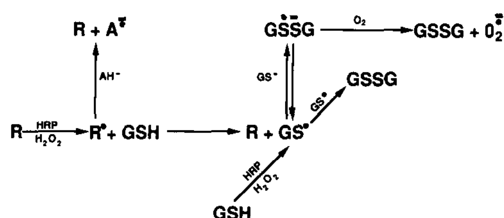

SCHEME 1. **A scheme for the reactions of ascorbate and glutathione with the phenoxyl radical of acetaminophen (R•).**

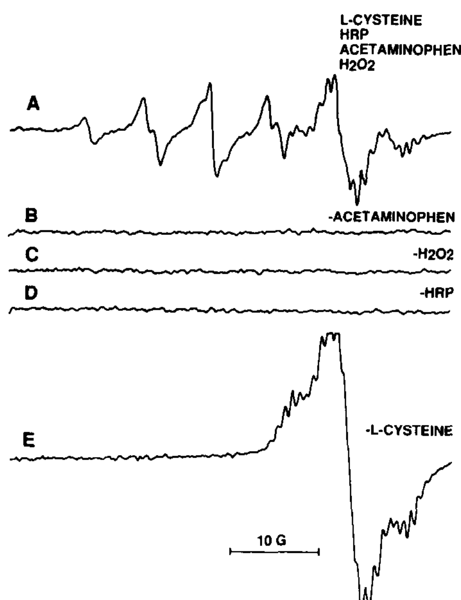

FIG. 1. *A*, the ESR spectrum of the cystine disulfide radical anion and the phenoxyl radical of acetaminophen generated in a system of cysteine (100 mM), horseradish peroxidase (*HRP*) type I (10 units/ml), hydrogen peroxide (25 mM), and acetaminophen (10 mM) in phosphate (0.05 M) buffer, pH 7.5, purged with nitrogen. *B*, same as in *A*, but in the absence of acetaminophen. *C*, same as in *A*, but in the absence of hydrogen peroxide. *D*, same as in *A*, but in the absence of horseradish peroxidase. *E*, same as in *A*, but in the absence of cysteine. The instrumental conditions were: 20 mW[1] microwave power, 0.53 G modulation amplitude, 0.5 s time constant, 6.25 G/min scan rate, and 5×10^4 receiver gain.

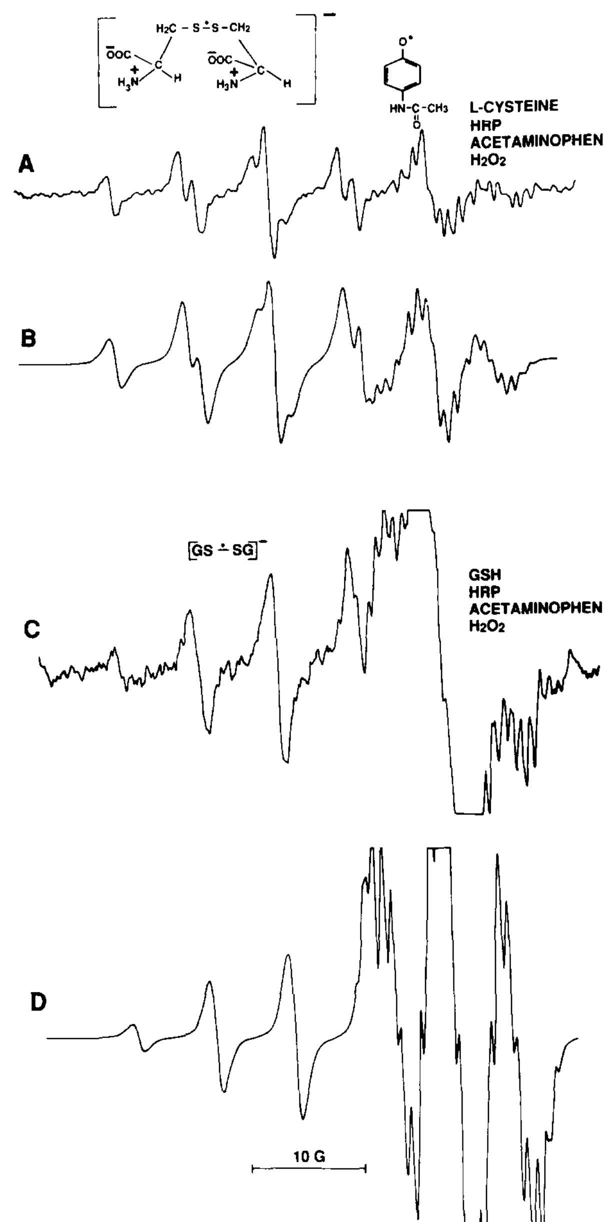

FIG. 2. *A*, the ESR spectrum of the cystine disulfide radical anion and the phenoxyl radical of acetaminophen generated in a system of cysteine (100 mM), horseradish peroxidase (*HRP*) type I (10 units/ml), hydrogen peroxide (25 mM), and acetaminophen (10 mM) in phosphate buffer (0.05 M), pH 7.5. The instrumental conditions were: 20 mW microwave power, 0.41 G modulation amplitude, 1 s time constant, 6.25 G/min scan rate, and 10×10^4 receiver gain. *B*, the computer simulation of the cystine disulfide radical anion. The derived hyperfine coupling constants are given in Table I. Line shape is 80% Lorentzian, 20% Gaussian, with a line width of 0.8 G. *C*, the ESR spectrum of the glutathione disulfide radical anion and the phenoxyl radical of acetaminophen generated in a system of glutathione (100 mM), horseradish peroxidase type I (10 units/ml), hydrogen peroxide (25 mM), and acetaminophen (10 mM) in phosphate (0.05 M) buffer, pH 7.5. The instrumental conditions were: 20 mW microwave power, 0.53 G modulation amplitude, 1 s time constant, 6.25 G/min scan rate, and 2.5×10^5 receiver gain. *D*, the computer simulation of the glutathione disulfide radical anion. The hyperfine coupling constants are given in Table I. The line shape is 80% Lorentzian, 20% Gaussian with a line width of 0.8 G.

Fig. 1. Fig. 1*A* shows the spectra of both the disulfide radical anion of cysteine and the phenoxyl radical of acetaminophen. The cysteinyl radical cannot be detected by direct ESR observation because of large anisotropy in the *g* factor (11), although it was recently reported that the thiyl radical can be detected in a low temperature matrix (12). The cysteinyl radical reacts with a cysteine thiolate anion to form the corresponding disulfide radical anion (Scheme 1). In the absence of acetaminophen (Fig. 1*B*), hydrogen peroxide (Fig. 1*C*), horseradish peroxidase (Fig. 1*D*), or cysteine (Fig. 1*E*) in the assay system, the formation of the cysteine disulfide radical anion was not detected. In Fig. 1*B*, the disulfide radical anion was not detected, although the thiyl free radical has been detected with spin trapping in similar incubations (13). Presumably, the disulfide radical anion is present at low, undetectable concentrations. In the absence of cysteine, the phenoxyl radical of acetaminophen is formed as shown in Fig. 1*E* (14).

The disulfide radical anion of cysteine was identified by the simulation (Fig. 2*B*) of the experimental spectrum (Fig. 2*A*) using the hyperfine coupling constants reported in Table I. The disulfide radical anion of glutathione obtained by the reaction of glutathione with the acetaminophen phenoxyl radical (Fig. 2*C*) was also simulated (Fig. 2*D*) by the hyperfine coupling constant reported in Table I. The formation of this radical is dependent upon the presence of glutathione, per-

[1] The abbreviation used is: mW, milliwatt(s).

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TABLE I
ESR parameters for the disulfide radical anions ($R\dot{S}\bar{S}R$) formed in horseradish peroxidase catalyzed reactions in fast-flow ESR experiments

Substrate	g values	Proton hyperfine coupling constants	
		2H	2H
Cysteine	2.0132	6.5	7.75
Glutathione	2.0133	7.0	7.0
Cystine[a]	2.0133	—[b]	8.50
Lipoic acid[c]	2.0129	7.8 (1H)	4.35 (1H) 1.45 (1H)

[a] Parameters obtained by the direct addition of an electron in a radiolysis experiment in a low-temperature matrix (15).
[b] Not resolved.
[c] Reference 19.

oxidase, hydrogen peroxide, and acetaminophen in the assay system (data not shown). The hyperfine coupling constants for the disulfide radical anions are given in Table I.

In the presence of ascorbate and acetaminophen in the peroxidase system, the phenoxyl radical of acetaminophen was not detected. Only the ascorbyl radical was formed (Fig. 3*A*). In the presence of glutathione (100 mM) and ascorbate (2 or 10 mM) in the peroxidase system (Fig. 3*B*), the amplitude of the ascorbyl radical spectrum was only slightly lower than that in the presence of ascorbate alone (Fig. 3*A*), and no glutathionyl disulfide radical anion could be detected. In the absence of ascorbate, only the phenoxyl radical of acetaminophen was detected, as expected (Fig. 3*C*). In the absence of acetominophen (Fig. 3*D*), peroxidase (Fig. 3*E*), or hydrogen peroxide (Fig. 3*F*), much lower concentrations of the ascorbyl radical were detected.

In the presence of glutathione in the peroxidase/acetaminophen system, oxygen consumption was rapid (Fig. 4*A*), suggesting that the oxygen consumption was due to reaction of oxygen with the disulfide radical anion of glutathione (Scheme 1). In the presence of ascorbate, this horseradish peroxidase-catalyzed reaction was strongly inhibited. There was no oxygen consumption in the presence of ascorbate alone (Fig. 4*C*). The oxygen consumption was dependent upon the presence of peroxidase, hydrogen peroxide, and glutathione in the assay system (Fig. 4).

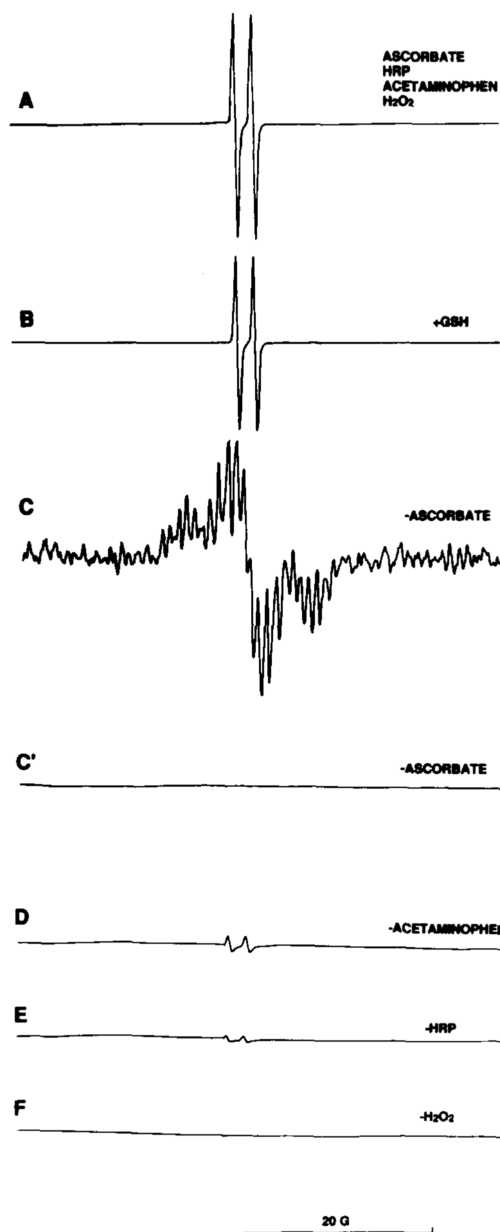

FIG. 3. *A*, the ESR spectrum of the ascorbyl radical generated in a system of acetaminophen (10 mM), hydrogen peroxide (25 mM), ascorbate (2 mM), and horseradish peroxidase (*HRP*) type I (10 units/ml) in phosphate (0.05 M) buffer, pH 7.4, purged with nitrogen. *B*, same as in *A*, but in the presence of glutathione (100 mM). *C*, same as in *A*, but in the absence of ascorbate, and at higher gain. *C'*, same as in *A*, but in the absence of ascorbate. *D*, same as in *A*, but in the absence of acetaminophen. *E*, same as in *A*, but in the absence of horseradish peroxidase. *F*, same as in *A*, but in the absence of hydrogen peroxide. The instrumental conditions for *A*, *B*, *C'*, *E*, and *F* were: 20 mW microwave power, 0.41 G modulation amplitude, 0.128 s time constant, 12.5 G/min scan rate, and 10×10^2 receiver gain; for *C*, 20 mW microwave power, 1.65 G modulation amplitude, 1 s time constant, 6.25 G/min scan rate, and 2×10^5 receiver gain; or *D*, 20 mW microwave power, 0.53 G modulation amplitude, 1 s time constant, 6.25 G/min scan rate, and 10×10^2 receiver gain.

DISCUSSION

Thiol pumping is suggested as one of the important mechanisms for the detoxification of free radical metabolites. Hence, the reaction of glutathione with free radical metabolites has been studied in several laboratories (2–8). The thiyl radical metabolite of glutathione detected as a DMPO radical adduct has been reported by Moldéus and co-workers (5, 6) in the thiol pumping of phenetidine and acetaminophen radical, but no ESR spectroscopic evidence has yet been presented for the formation of the disulfide radical anion. Fig. 1 clearly demonstrates the formation of the cysteine disulfide radical anion in the reaction between the acetaminophen phenoxyl radical and cysteine. The formation of this radical is dependent upon the presence of acetaminophen, horseradish peroxidase, hydrogen peroxide, and cysteine. The *g* values for the disulfide radicals shown in Table I are characteristic of these sulfur-centered radicals. These values agree with the *g* values for the disulfide radical anions produced in a low-temperature matrix (15). For glutathione, the hyperfine coupling constants for the β-methylene protons (4H) are very well resolved, but the α-methine protons (2H) are too small to be observed. For cysteine, the four methylene protons were inequivalent and in pairs, giving hyperfine coupling constants of 6.5 and 7.75 G. This was anticipated because these β-methylene protons are bonded to a carbon atom which is adjacent to an asymmetric α-carbon. Hence the methylene protons are inequivalent with respect to the radical center (16–18). With glutathione disulfide radical anion, this inequivalence is much less than the line width, and the four methylene hydrogens are apparently equivalent. In the solid phase, only two of the four hyperfine coupling constants for the four

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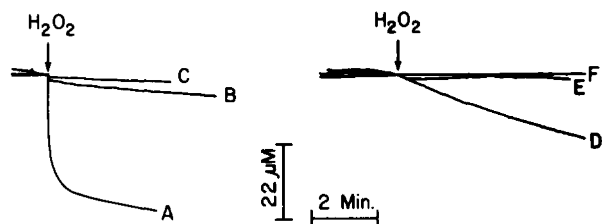

FIG. 4. *A*, the oxygen consumption curve by a 2-ml incubation containing acetaminophen (0.25 mM), horseradish peroxidase type VI (25 μg/ml), hydrogen peroxide (0.125 mM), and glutathione (3 mM) in phosphate (0.05 M) buffer, pH 7.4, containing diethylenetriamine pentaacetic acid (0.5 mM) at 30 °C. *B*, same as in *A*, but in the presence of ascorbate (1 mM). *C*, same as in *A*, but in the presence of ascorbate (1 mM) and absence of glutathione. *D*, same as in *A*, but in the absence of horseradish peroxidase. *E*, same as in *A*, but in the absence of acetaminophen. *F*, same as in *A*, but in the absence of hydrogen peroxide.

methylene hydrogens of cysteine disulfide radical anion were resolved (15). For lipoic acid, in the liquid phase, the coupling constants for only three of four protons were resolved (19).

It is clearly demonstrated that ascorbate reacts with the acetaminophen phenoxyl radical completely on the millisecond time scale (Fig. 3). There is no trace of the acetaminophen phenoxyl radical; only the ascorbyl radical was detected (a^H = 1.8 G). In the presence of glutathione or cysteine, the corresponding disulfide radical (Figs. 1 and 2) is present with the acetaminophen phenoxyl radical. This shows that ascorbate reacts with the acetaminophen radical more rapidly than does glutathione. Thus, ascorbate will be the more effective reducing biochemical than glutathione. This is significant because in human liver the glutathione-to-ascorbate ratio is about 6 (20, 21). Thus, it suggests a dominant physiological role for ascorbate over glutathione, because even at a glutathione-to-ascorbate ratio of 10 or even 50, only the ascorbyl radical was detected. The ascorbyl radical is known to be formed by the ascorbate reduction of the thiyl radical (22), but under the experimental conditions employed in this study, this is unlikely to have occurred because in the presence of ascorbate and glutathione in the peroxidase system, no acetaminophen phenoxyl radical can be detected, whereas in the presence of glutathione, the acetaminophen phenoxyl radical is not completely reduced (Fig. 3*C*). These results imply that in the presence of ascorbate the glutathione thiyl free radical does not form in significant amounts and, therefore, cannot be the source of the ascorbate semidione free radical.

Ascorbate reduction of the disulfide radical anion is not known; hence, the ascorbyl radical could not have formed by this pathway. Fig. 3*D* shows that the ascorbyl radical was formed in relatively insignificant quantities by the peroxidase system. In the presence of both glutathione and ascorbate, the amplitude of the ESR spectrum of the ascorbyl radical was only slightly decreased when compared to the system containing ascorbate alone (Fig. 3, *A* and *B*). This suggests that in the presence of glutathione, the thiol is not very competitive with ascorbate for the reduction of the acetaminophen radical. This is also illustrated in the oxygen uptake measurements (Fig. 4). In the presence of glutathione and ascorbate, the oxygen uptake is insignificant compared with the system containing only glutathione. In the presence of ascorbate, but in the absence of glutathione, there is no oxygen consumption because the ascorbate semidione free radical reacts with oxygen very slowly (23). The oxygen consumption is expected in the presence of glutathione due to the reactions of the free radical metabolites of glutathione with oxygen (24, 25).

The results presented above clearly show that ascorbate may be a better reductant than glutathione for the phenoxyl radical of acetaminophen. Only in cells where the cytoplasm contains low levels of ascorbate and high levels of glutathione (26) can the latter play an important role in the detoxification mechanism. In the lipid matrix other biochemical reducing agents such as α-tocopherol are probably more important than the membrane-impermeable ascorbate.

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