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 phenoxy 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 phenoxy radical of acetaminophen to regenerate acetaminophen and form the thiol radical of glutathione. This thiol radical reacts with the thiolate anion of glutathione to form the disulfide radical anion, which was detected and characterized by ESR spectroscopy. The reaction of oxygen with the disulfide radical anion is fast and was used to identify this reaction product as GSSG. In the presence of both ascorbate and higher concentrations of glutathione, the reaction with ascorbate is dominant.

Glutathione and ascorbate can also be used to reduce the phenoxyl radical of acetaminophen. 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 phenoxy 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 phenoxy 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 thiol 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.

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 K-109 spectrometer equipped with a TM109 cavity at room temperature. Reagents were prepared in two 1-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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Glutathione and Ascorbate Reduction of the Acetaminophen Radical

**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 cysteine 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 microwave power, 0.53 G modulation amplitude, 0.5 s time constant, 6.25 G/min scan rate, and $5 \times 10^4$ receiver gain.

The disulfide radical anion of cysteine was identified by the simulation (Fig. 2B) of the experimental spectrum (Fig. 2A) using the hyperfine coupling constants reported in Table I.

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

The disulfide radical anion of glutathione obtained by the reaction of glutathione with the acetaminophen phenoxyl radical (Fig. 2C) was also simulated (Fig. 2D) by the hyperfine coupling constant reported in Table I. The formation of this radical is dependent upon the presence of glutathione, per-
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. 3A). In the presence of glutathione (100 mM) and ascorbate (2 or 10 mM) in the peroxidase system (Fig. 3B), the amplitude of the ascorbyl radical spectrum was only slightly lower than that in the presence of ascorbate alone (Fig. 3A), 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. 3C). In the absence of acetaminophen (Fig. 3D), peroxidase (Fig. 3E), or hydrogen peroxide (Fig. 3F), much lower concentrations of the ascorbyl radical were detected.

In the presence of glutathione in the peroxidase/acetaminophen system, oxygen consumption was rapid (Fig. 4A), suggesting that the oxygen consumption was due to reaction of oxygen with the disulfide radical anion of glutathione (Scheme I). 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. 4C). The oxygen consumption was dependent upon the presence of peroxidase, hydrogen peroxide, and glutathione in the assay system (Fig. 4).

**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 Moldau and co-workers (5, 6) and 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 \( \beta \)-methylene protons (4H) are very well resolved, but the \( \alpha \)-methylene protons (2H) are too small to be observed. For cysteine, the four \( \alpha \)-methine protons were inequivalent and in pairs, giving hyperfine coupling constants of 6.5 and 7.75 G. This was anticipated because these \( \beta \)-methylene protons are bonded to a carbon atom which is adjacent to an asymmetric \( \alpha \)-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

**TABLE I**

| Substrate  | g values | Proton hyperfine coupling constants |
|------------|----------|------------------------------------|
|            |          | 2H  | 2H               |
| Cysteine   | 2.0132   | 6.5 | 7.75             |
| Glutathione| 2.0133   | 7.0 | 7.0              |
| Cystine    | 2.0133   | --  | 8.50             |
| Lipoic acid| 2.0129   | 7.8 (1H) | 4.35 (1H) |
|           |          |     | 1.45 (1H)        |

* Parameters obtained by the direct addition of an electron in a radiolysis experiment in a low-temperature matrix (15).

* Not resolved.

* Reference 19.
The presence of ascorbate (1 mM), horseradish peroxidase, and hydrogen peroxide (0.125 mM), and glutathione (3 mM) in phosphate (0.05 mM) buffer, pH 7.4, containing dithionylethramine pentacetic acid (0.5 mM) at 30°C, C, same as in A, but in the absence of acetaminophen radical. This is also illustrated in the oxygen uptake measurements (Fig. 4). In the presence of glutathione, the thiol-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 pathway does not form in significant amounts and, therefore, cannot be the source of the ascorbate semidione free radical.

Acknowledgment—We thank L. Harman for excellent technical assistance.

REFERENCES

1. Fehle, L. (1982) in Free Radicals in Biology (Pyror, W. A., ed) Vol. 5, pp. 225–234, Academic Press, New York.
2. Ohnishi, T., Yamazaki, H., Iyanagi, T., Nakamura, T., and Yamazaki, I. (1969) Biochim. Biophys. Acta 172, 357–369.
3. Kataoka, N., Shibata, S., Imamura, A., Kawazoe, Y., Chihara, G., and Nagata, C. (1967) Chem. Pharm. Bull. (Tokyo) 15, 220–225.
4. Horumato, M., Inuzuka, S., and Sugimoto, T. (1967) Cancer Res. 27, 1378–1383.
5. Ross, D., Albano, E., Nilsson, U., and Moleud, P. (1984) Biochem. Biophys. Res. Commun. 125, 109–115.
6. Ross, D., Norbeck, K., and Moldeus, P. (1985) J. Biol. Chem. 260, 15028–15032.
7. Ellis, T. E., Mason, R. P., and Sivarajah, K. (1985) J. Biol. Chem. 260, 1601–1607.
8. Hoffman, M. Z., and Hayon. E. (1972) J. Am. Chem. Soc. 94, 7950–7957.
9. Ross, D. (1988) Pharmacol. Ther. 37, 231–249.
10. West, P. R., Harman, L. S., Joseph, P. D., and Mason, R. P. (1984) Biochem. Pharmacol. 33, 2923–2936.
11. Symons, M. C. R. (1974) J. Chem. Soc. Perkin Trans. II 1618–1620.
12. Sevilla, M. D., Becker, D., Swarts, S., and Herrington, J. (1987) Biochem. Biophys. Res. Commun. 144, 1037–1042.
13. Fukushima, L. S., Morgan, C., and Mason, R. P. (1964) J. Biol. Chem. 259, 5606–5611.
14. Fischer, V., Harman, L. S., West, P. R., and Mason, R. P. (1986) Chem.-Biol. Interact. 60, 115–127.
15. Rao, D. R. N., Symons, M. C. R., and Stephenson, J. M. (1983) J. Chem. Soc. Perkin Trans. II, 727–730.
16. Kreilick, R. W., Becher, J., and Ulman, E. F. (1989) J. Am. Chem. Soc. 111, 5121–5124.
17. Gilbert, B. C., Larkin, J. P., and Norman, R. O. C. (1972) J. Chem. Soc. Perkin Trans. II, 1272–1279.
18. Seay, R. C., Harman, L. S., West, P. R., and Mason, R. P. (1985) J. Am. Chem. Soc. 107, 3401–3406.
19. Gilbert, B. C., Lase, H. A. H., Norman, R. O. C., and Seay, R. C. (1975) J. Chem. Soc. Perkin Trans. II, 892–900.
20. Altomare, V., Vendemiale, G., and Albano, O. (1988) Life Sci. 43, 991–998.
21. Yavorsky, M., Almaden, P., and King, C. G. (1934) J. Biol. Chem. 106, 525–529.
22. Forni, L. G., Monig, J., Mora-Arellano, V. O., and Willson, R. L. (1983) J. Chem. Soc. Perkin Trans. II, 961–965.
23. Biekie, B. H. J., Richter, H. W., and Chan, P. C. (1975) Ann. N. Y. Acad. Sci. 258, 231–237.
24. Sevilla, M. D., Yan, M., and Becker, D. (1988) Biochem. Biophys. Res. Commun. 155, 405–410.
25. Saez, G., Thornalley, P. J., Hill, H.A. O., Hems, R., and Bannister, J. V. (1982) Biochem. Biophys. Aec 719, 24–31.
26. Schreiber, J., Fourman, G. L., Hughse, M. F., Mason, R. P., and Eling, T. E. (1989) J. Biol. Chem. 264, 7536–7543.
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J. Biol. Chem. 1990, 265:844-847.

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