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The Fate of the Oxidizing Tyrosyl Radical in the Presence of Glutathione and Ascorbate

IMPLICATIONS FOR THE RADICAL SINK HYPOTHESIS*

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Cellular systems contain as much as millimolar concentrations of both ascorbate and GSH, although the GSH concentration is often 10-fold that of ascorbate. It has been proposed that GSH and superoxide dismutase (SOD) act in a concerted effort to eliminate biologically generated radicals. The tyrosyl radical (Tyr$^{\bullet}$) generated by horseradish peroxidase in the presence of hydrogen peroxide can react with GSH to form the glutathione thiyl radical (GS$^{\bullet}$). GS$^{\bullet}$ can react with the glutathione anion (GS^{-}) to form the disulfide radical anion (GSSG$^{\bullet-}$). This highly reactive disulfide radical anion will reduce molecular oxygen, forming superoxide and glutathione disulfide (GSSG). In a concerted effort, SOD will catalyze the dismutation of superoxide, resulting in the elimination of the radical. The physiological relevance of this GSH/SOD concerted effort is questionable. In a tyrosyl radical-generating system containing ascorbate (100 μM) and GSH (8 mM), the ascorbate nearly eliminated oxygen consumption and diminished GS$^{\bullet}$ formation. In the presence of ascorbate, the tyrosyl radical will oxidize ascorbate to form the ascorbate radical. When measuring the ascorbate radical directly using fast-flow electron spin resonance, only minor changes in the ascorbate radical electron spin resonance signal intensity occurred in the presence of GSH. These results indicate that in the presence of physiological concentrations of ascorbate and GSH, GSH is not involved in the detoxification pathway of oxidizing free radicals formed by peroxidases.

The "radical sink hypothesis" proposed by Winterbourn (1, 2) suggests a concerted antioxidant interaction between GSH and superoxide dismutase (SOD).[1] In this proposal, biologically generated radicals (R$^{\bullet}$) oxidize GSH to form thiyl radicals (GS$^{\bullet}$) (Equation 1). As pointed out by Winterbourn (1, 2), this oxidizing thiyl radical is not biologically benign and can undergo other potentially harmful reactions. The reaction of GS$^{\bullet}$ with the glutathione anion (GS^{-}) produces the glutathione disulfide radical anion (GSSG$^{\bullet-}$) (Equation 2), which, in turn, reduces molecular oxygen, forming superoxide ($O_2^{\bullet-}$) and glutathione disulfide (GSSG) (Equation 3). In a concerted effort, SOD will catalyze the dismutation of superoxide, terminating the biologically generated free radical (Equation 4). This proposal has been shown to be thermodynamically feasible (3).

$$R^{\bullet} + GSH \rightleftharpoons RH + GS^{\bullet} \quad \text{(Eq. 1)}$$

$$GS^{\bullet} + GS^{-} \rightleftharpoons GSSG^{\bullet-} \quad \text{(Eq. 2)}$$

$$GSSG^{\bullet-} + O_2 \rightarrow GSSG + O_2^{\bullet-} \quad \text{(Eq. 3)}$$

$$2\,O_2^{\bullet-} + 2H^{+} \xrightarrow{SOD} O_2 + H_2O_2 \quad \text{(Eq. 4)}$$

However, the physiological relevance of GSH as an antioxidant has been questioned (4–6). Based on known rate constants, Wardman (4) has estimated the fraction of thiyl radicals that are not conjugated as GSSG$^{\bullet-}$ or GSOO$^{\bullet}$ (from the reaction of GS$^{\bullet}$ with O_2). He found that in well oxygenated tissue (40 μM O_2) at pH 7.4 and with 2 mM GSH, ~80% of the thiyl radicals are not conjugated, indicating that GS$^{\bullet}$, not GSSG$^{\bullet-}$, is the significant radical species under these conditions. In conjunction with this work, Wardman estimated the fraction of thiyl radicals which leads to superoxide formation in the presence of ascorbate. Based on the published rate constants (7, 8) for the reaction of ascorbate with GS$^{\bullet}$, he found that in well oxygenated tissues (40 μM O_2) at pH 7.4 with 1 mM GSH and 0.05 mM ascorbate, only 3% of the thiyl radicals follow the superoxide pathway. In addition, ascorbate abolishes O_2 consumption that occurs from the reaction of GSH with enzymatically generated radical metabolites of acetaminophen (9), clozapine (10), crystal violet (11), 17β-estradiol (12), and phenolphthalein (13). When these radical metabolites are generated in the presence of ascorbate, the ascorbate radical is generated via oxidation by the metabolite radical, and the concentration of the ascorbate radical is minimally affected by the presence of GSH (9–13). Work done with the VP-16 phenoxyl radical showed that, in a system that contained ascorbate and GSH, the ascorbate is oxidized prior to the oxidation of GSH (5). Goldman *et al.* (6) discussed the interaction of two phenoxyl radicals (from *p*-cresol and VP-16) and intercellular reductants (ascorbate, GSH, and NAD(P)H). Rate constants for relevant reactions led them to conclude that GSH should be well protected from oxidation by *p*-cresol phenoxyl radical unless the concentration of ascorbate drops below 1/600th that of GSH.

In view of the absence of experimental data exploring the role of physiological concentrations of ascorbate and GSH in the fate of biologically generated free radicals, we have initi-

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[1] The abbreviations used are: SOD, superoxide dismutase; GS$^{\bullet}$, glutathione thiyl radical; GSSG$^{\bullet-}$, glutathione disulfide radical anion; $O_2^{\bullet-}$, superoxide; ESR, electron spin resonance; DTPA, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide.

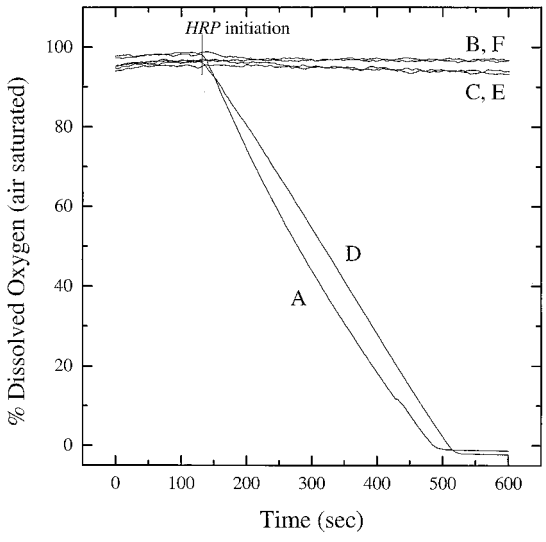


FIG. 1. **Oxygen consumption by the horseradish peroxidase system with 8 mM glutathione.** The system contained horseradish peroxidase (*HRP*) (15 units/ml = 13.9 μg/ml), tyrosine (2 mM), hydrogen peroxide (36 μM), and GSH (8 mM). *A*, complete system with HRP, tyrosine, H_2O_2, and GSH. *B*, as in *A*, but no HRP. *C*, as in *A*, but no tyrosine. *D*, as in *A*, but no H_2O_2. *E*, as in *D*, but catalase (6,500 units/ml) was added 1 min before HRP. *F*, as in *A*, but no GSH.

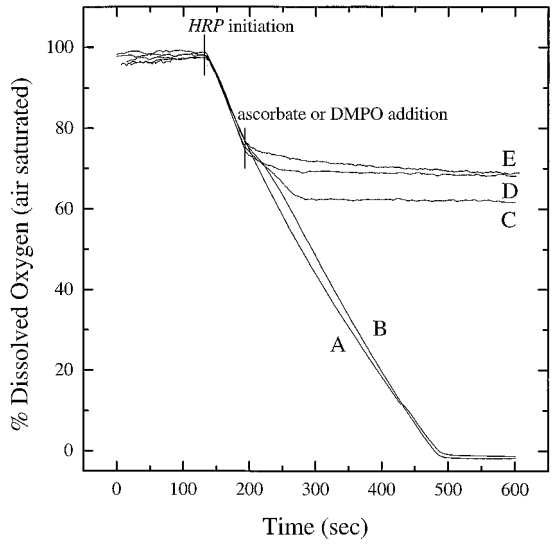


FIG. 2. **Oxygen consumption by the horseradish peroxidase system with 8 mM glutathione was eliminated upon addition of 100 μM ascorbate.** The system contained horseradish peroxidase (*HRP*) (15 units/ml = 13.9 μg/ml), tyrosine (2 mM), hydrogen peroxide (36 μM), and GSH (8 mM). Addition of ascorbic acid or DMPO was done 1 min after the HRP initiation of tyrosyl radicals in order to establish an initial rate of oxygen consumption. *A*, complete system with HRP, tyrosine, H_2O_2, and GSH. *B*, as in *A*, but 25 μM ascorbic acid added 1 min after HRP initiation. *C*, as in *A*, but 50 μM ascorbic acid added 1 min after HRP initiation. *D*, as in *A*, but 100 μM ascorbic acid added 1 min after HRP initiation. *E*, as in *A*, but 100 mM DMPO added 1 min after HRP initiation.

ated an investigation using horseradish peroxidase in the presence of hydrogen peroxide to generate tyrosyl radicals. The fate of the tyrosyl radical was determined in the presence of varying ratios of ascorbate and GSH using oxygen consumption measurements and electron spin resonance (ESR) spectroscopy.

MATERIALS AND METHODS

Reagents—Ascorbic acid, diethylenetriaminepentaacetic acid (DTPA), GSH, horseradish peroxidase type VI-A (EC 1.11.1.7), and tyrosine were purchased from Sigma and used as received. The quoted horseradish peroxidase activity is based on the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) assay. Hydrogen peroxide (H_2O_2) was purchased from Fisher. The H_2O_2 concentration was verified using the UV absorption at 240 nm (ϵ = 43.6 M^{-1}). Catalase (from beef liver, 65,000 units/mg suspension in water) (EC 1.11.1.6) and SOD (from bovine erythrocytes, 5000 units/mg of lyophilizate) (EC 1.15.1.1) were purchased from Boehringer Mannheim and used as received. The 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin trap was purchased from Aldrich, vacuum-distilled twice, and stored under nitrogen at −70 °C until needed. All reactions used a 100 mM phosphate buffer at pH 7.4, Chelexed overnight, and 100 μM DTPA was added after Chelexing. Due to the high concentration of GSH (8 mM) used in most experiments, all pH values were carefully checked and adjusted as required prior to initiation of tyrosyl radical generation. Stock solutions of reagents were made fresh daily and stored on ice while experiments were being performed. The stock solutions were made up as follows: horseradish peroxidase in buffer, H_2O_2 in deionized water, tyrosine in buffer, GSH in deionized water, ascorbic acid in deionized water, and SOD in buffer. Catalase was used as received, and DMPO was used undiluted.

Oxygen Consumption Experiments—Oxygen consumption measurements were made using a Clark-type oxygen electrode fitted to a 1.8-ml Gilson sample cell and monitored by a Yellow Springs Instrument Company model 53 oxygen monitor (Yellow Springs, OH). The reagents were added in the following order: tyrosine/buffer stock solution, GSH stock solution, H_2O_2 stock solution, and SOD stock solution (when used). Then, after establishing a 2-min base-line measurement, horseradish peroxidase stock solution was added to initiate tyrosyl radical generation. In experiments that contained ascorbate, ascorbic acid stock solution was added either 1 min prior to horseradish peroxidase or 1 min after horseradish peroxidase. In experiments that contained DMPO, undiluted DMPO was added either 1 min prior to horseradish peroxidase or 1 min after horseradish peroxidase.

ESR Experiments—ESR experiments were carried out on a Bruker ER-200D ESR spectrometer interfaced to an IBM-compatible computer. All experiments were done at room temperature. Fast-flow ESR experiments which detected the tyrosyl radical were done using a TM_{110} resonator equipped with a 17-mm quartz, mixing flat cell (~225-μl volume) designed for fast-flow measurements (Wilmad, NJ) (14). This mixing flat cell had a post-mixing dead volume of approximately 300 μl, which, when flowing at 60 ml/min, resulted in a post-mixing observation time of approximately 300 ms. Fast-flow ESR measurements of the ascorbate radical were done using a Bruker dielectric mixing resonator with a 1-μl active volume. Spin-trapping experiments were done using a standard 17-mm quartz flat cell (~225-μl volume) mounted in the TM_{110} resonator. Spin-trapping samples were prepared, mixed, and then aspirated into the flat cell with ESR data acquisition started approximately 15 s after mixing.

RESULTS

Oxygen Consumption in the Presence of Ascorbate—The role of GSH in the proposed concerted action of GSH and SOD is to channel electrons to molecular oxygen through the formation of the highly reducing disulfide radical anion ($GSSG^{\cdot-}$). As outlined in Equations 1–3, where $R^{\cdot}$ = tyrosyl radical, the formation of $GS^{\cdot}$ will consume molecular oxygen. For this reason we have made oxygen consumption measurements in the presence and absence of ascorbate, GSH, and SOD. As reported (15), oxygen was consumed during the horseradish peroxidase-dependent oxidation of tyrosine in the presence of GSH (Fig. 1, *A*). Oxygen consumption was strictly dependent on the presence of horseradish peroxidase (Fig. 1, *B*), tyrosine (Fig. 1, *C*), and GSH (Fig. 1, *F*). The omission of H_2O_2 decreased the rate only slightly (Fig. 1, *D*). Hydrogen peroxide is obligatory to support the oxygen consumption as shown by catalase inhibition (Fig. 1, *E*). Fig. 2 shows the oxygen consumption data for a system that contained horseradish peroxidase (15 U/ml), tyrosine (2 mM), H_2O_2 (36 μM), GSH (8 mM), and varying concentrations of ascorbate. The post-addition (1 min after horseradish peroxidase-initiation) of 25 μM ascorbate (Fig. 2, *B*) had only a minor effect on the rate of oxygen consumption. The post-addition of 50 μM ascorbate (Fig. 2, *C*) inhibited the rate and extent of oxygen consumption. The post-addition of 100 μM ascorbate (Fig. 2, *D*) nearly eliminated oxygen consumption. Identical results were observed when ascorbate was added 1 min before

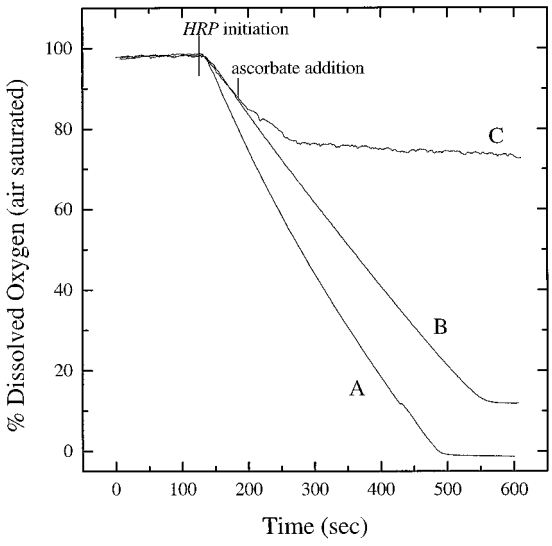


FIG. 3. **Oxygen consumption by the horseradish peroxidase system with 8 mM GSH was decreased in the presence of SOD.** The system contained horseradish peroxidase (*HRP*) (15 units/ml = 13.9 μg/ml), tyrosine (2 mM), hydrogen peroxide (36 μM), and GSH (8 mM). *A*, complete system with HRP, tyrosine, H_2O_2, and GSH. *B*, as in *A*, but SOD (750 units/ml) was added 2 min before HRP. *C*, as in *B*, but 25 μM ascorbic acid was added 1 min after HRP initiation.

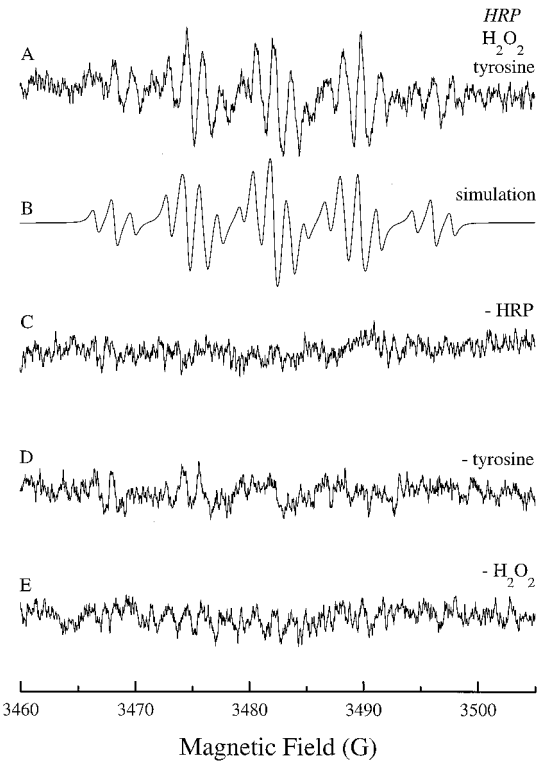


FIG. 4. **Fast-flow electron spin resonance of the enzymatically generated tyrosyl radical.** The system contained horseradish peroxidase (*HRP*) (15 units/ml = 13.7 μg/ml), tyrosine (~2.1 mM), and hydrogen peroxide (1.9 mM). Equal volumes of tyrosine/H_2O_2 and HRP in pH 7.4 phosphate buffer (50 mM phosphate, 50 μM DTPA) solutions were mixed (at a flow rate of 60 ml/min total volume), and ESR observation occurred ~300 ms after mixing. The solvent reservoirs for the two solutions were immersed in a water bath held at 37 °C. *A*, complete system with HRP, tyrosine, and H_2O_2. Deoxygenation by nitrogen bubbling prior to mixing produced an ESR spectrum essentially identical with *A* (data not shown). *B*, optimized computer simulation using literature values (21) for the ESR parameters of the tyrosyl radical. *C*, as in *A*, but no HRP. *D*, as in *A*, but no tyrosine. *E*, as in *A*, but no H_2O_2. Instrument conditions: microwave frequency, 9.7 GHz; microwave power, 20 milliwatts; resonator, TM_{110} with 17-mm flat cell; modulation amplitude (100 kHz), 0.5 G; receiver gain, 1.6×10^6; time constant, 5.0 s; sweep rate, 0.0333 G/sec.

the addition of horseradish peroxidase (data not shown).

If all GSH-dependent superoxide (Equation 3) underwent nonenzymatic bimolecular dismutation, then SOD would have no effect on the oxygen uptake. Fig. 3 shows that the rate of oxygen consumption in the absence of SOD (Fig. 3, *A*) was higher than in the presence of SOD (Fig. 3, *B*), indicating that the superoxide generated was involved in additional oxygen-consuming chemistry (16–18). In the presence of SOD, 25 μM ascorbate (Fig. 3, *C*) inhibited the rate and extent of oxygen consumption, whereas in the absence of SOD, 25 μM ascorbate had a minor effect on the rate and extent of oxygen consumption (Fig. 2, *B*), indicating that, in the absence of SOD, the higher rate of oxygen consumption (and radical production) probably depleted the relatively low ascorbate concentration. Under conditions of lower GSH concentrations (<2 mM), SOD actually stimulates oxygen consumption in this system (15), so the effect of SOD is quite complex.

Direct ESR Observation of Enzymatically Generated Tyrosyl Radical—The enzymatically generated, free tyrosyl radical has been directly observed for the first time using fast-flow ESR (Fig. 4). Tyrosine is oxidized by horseradish peroxidase compound I at a rate of 5.0×10^4 M^{-1} s^{-1} (19) and compound II at a rate of 1.1×10^3 M^{-1} s^{-1} (20). The observed tyrosyl radical ESR spectrum (Fig. 4, *A*) was dependent on the presence of horseradish peroxidase (Fig. 4, *C*), tyrosine (Fig. 4, *D*), and H_2O_2 (Fig. 4, *E*). The ESR spectrum can be explained based on rotational exchange effects related to the hindered internal rotation of the $ArCH_2$-$CHNH_2CO_2^-$ bond (21). Deaeration of the solutions by nitrogen bubbling prior to tyrosyl radical generation produced ESR spectra essentially identical to those shown in Fig. 4 (data not shown). This tyrosyl radical-generating system did not consume oxygen (Fig. 1, *F*), implying that no oxygen-centered radicals were formed. Tyrosyl radical reacts with oxygen with a rate constant less than 10^3 M^{-1} s^{-1} (22, 23).

Direct ESR Observation of Ascorbate Radical Anion—When the tyrosyl radical was formed in the presence of ascorbate (pH 7.4), the ascorbate was rapidly oxidized by the tyrosyl radical to the ascorbate radical ($k = 4.4 \times 10^8$ M^{-1} s^{-1}) (22). Ascorbate is readily oxidized by a number of phenoxyl radicals (~10^8 M^{-1} s^{-1}) (24). The fast-flow ESR spectrum of the ascorbate radical generated from the tyrosyl radical-generating horseradish peroxidase system is shown in Fig. 5, *A*. The intensity of the ascorbate radical was decreased only slightly in the presence of 8 mM GSH (Fig. 5, *B*). The ESR spectrum of the ascorbate radical was dependent on the presence of horseradish peroxidase (Fig. 5, *C*), tyrosine (Fig. 5, *D*), H_2O_2 (Fig. 5, *E* and *F*), and ascorbate (Fig. 5, G). The ascorbate radical ESR signal observed in the absence of tyrosine (Fig. 5, *D*) is a result of direct oxidation of ascorbate by horseradish peroxidase compound I (25) and was only slightly greater than the nonenzymatic ascorbate radical formation (Fig. 5, *C*). For this reason ascorbate has been referred to as a "sluggish" substrate of horseradish peroxidase compound I (26). The stimulation of ascorbate radical by tyrosine (Fig. 5, *A*) demonstrated that tyrosine was oxidized by horseradish peroxidase in the presence of ascorbate and that ascorbate did not merely inhibit tyrosine oxidation.

Because ascorbate (AH^-) will react with both the tyrosyl radical ($Tyr^{\bullet}$) (Equation 5) and $GS^{\bullet}$ (Equation 6) to form the ascorbate radical ($A^{\bullet-}$), it is unclear from the results in Fig. 5 whether ascorbate inhibited $GS^{\bullet}$ formation by reacting with all of the tyrosyl radical or if ascorbate was oxidized by $GS^{\bullet}$ and, hence, eliminated oxygen consumption (Equation 3). As shown by oxygen consumption measurements, 100 mM DMPO completely inhibited oxygen consumption (Fig. 2, *E*), indicating

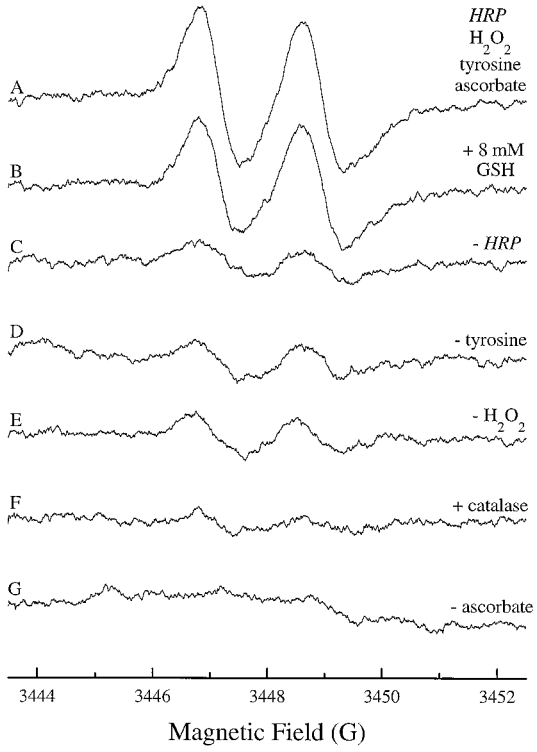


FIG. 5. **Fast-flow electron spin resonance of the ascorbate radical generated during the oxidation of tyrosine by horseradish peroxidase/hydrogen peroxide.** The system contained horseradish peroxidase (*HRP*) (15 units/ml = 11.4 μg/ml), tyrosine (2 mM), hydrogen peroxide (1.9 mM), and ascorbate (100 μM). Equal volumes of tyrosine/H_2O_2/ascorbate and HRP in air-saturated, pH 7.4, phosphate buffer (100 mM phosphate, Chelex-treated, 100 μM DTPA) solutions were mixed immediately before entering the dielectric resonator at a flow rate of 6 ml/min. All measurements were done at room temperature. *A*, complete system with HRP, tyrosine, H_2O_2, and ascorbate. *B*, as in *A*, with 8 mM GSH added. *C*, as in *A*, but no HRP. *D*, as in *A*, but no tyrosine. *E*, as in *A*, but no H_2O_2. *F*, as in *E*, but catalase (6500 units/ml) added. *G*, as in *A*, but no ascorbate. Instrument conditions: microwave frequency, 9.6 GHz; microwave power, 5 milliwatts; resonator, dielectric mixer; modulation amplitude (100 kHz), 1.6 G; receiver gain, 1.25×10^5; time constant, 5.0 s; sweep rate, 0.0333 G/s.

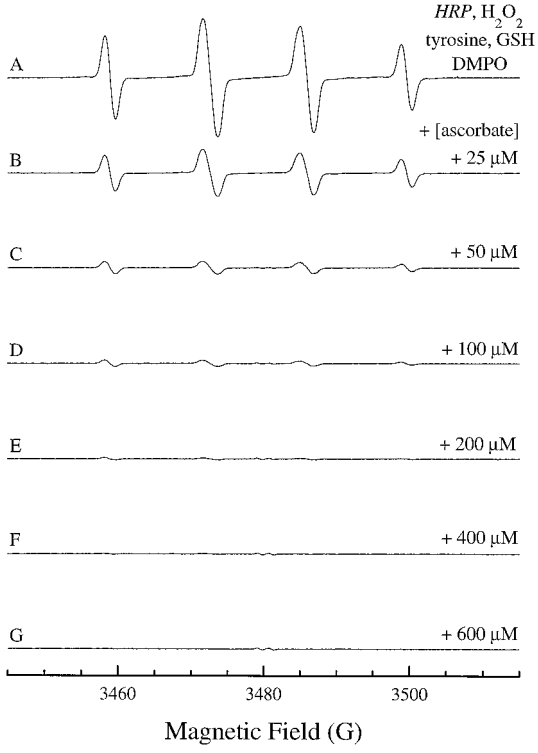


FIG. 6. **Spin trapping of GS$^\bullet$ in the presence of ascorbate.** GS$^\bullet$ was generated by the horseradish peroxidase (*HRP*) system which contained HRP (15 units/ml = 11.4 μg/ml), tyrosine (2 mM), hydrogen peroxide (36 μM), and GSH (8 mM) and trapped using 5,5-dimethyl-1-pyrroline *N*-oxide (100 mM). *A*, the ESR spectrum of the DMPO/$^\bullet$SG adduct detected with a complete system containing *HRP*, tyrosine, H_2O_2, GSH, and DMPO. The DMPO/$^\bullet$GS adduct formation was initiated by HRP in the presence of 25 μM ascorbate (*B*), 50 μM ascorbate (*C*), 100 μM ascorbate (*D*), 200 μM ascorbate (*E*), 400 μM ascorbate (*F*), and 600 μM ascorbate (*G*). Instrument conditions: microwave frequency, 9.7 GHz; microwave power, 20 milliwatts; resonator, TM_{110} with 17-mm flat cell; modulation amplitude (100 kHz), 1.6 G; receiver gain, 1×10^4; time constant, 80 ms; sweep rate, 0.889 G/s.

that DMPO efficiently traps all GS$^\bullet$ generated (Equation 7).

$$Tyr^\bullet + AH^- \rightarrow Tyr + A^{\bullet-} \quad \text{(Eq. 5)}$$

$$GS^\bullet + AH^- \rightarrow GSH + A^{\bullet-} \quad \text{(Eq. 6)}$$

$$GS^\bullet + DMPO \rightarrow DMPO/^\bullet SG \quad \text{(Eq. 7)}$$

If DMPO is included in the fast-flow ESR experiments designed to detect the ascorbate radical and if GS$^\bullet$ is formed, DMPO will trap GS$^\bullet$ ($k = 2.6 \times 10^8$ M^{-1} s^{-1}) (27) and decrease the intensity of the fast-flow ascorbate radical ESR signal by eliminating the ascorbate radical generated in Equation 6. When DMPO (100 mM) was added to the tyrosyl radical-generating horseradish peroxidase system with 100 μM ascorbate, there was no change in intensity of the ascorbate radical ESR signal (data not shown), indicating that, under fast-flow conditions, the ascorbate radical was formed only by reaction with the tyrosyl radical and not by reaction with GS$^\bullet$.

Spin Trapping of GS$^\bullet$ in the Presence of Ascorbate and the Direct Reduction of the DMPO/$^\bullet$SG Adduct by Ascorbate—The glutathione thiyl radical (GS$^\bullet$) was generated as a result of the horseradish peroxidase/H_2O_2-dependent formation of the tyrosyl radical as shown by ESR spin trapping with DMPO. DMPO was shown to completely inhibit oxygen consumption in a GS$^\bullet$-forming system (Fig. 2, *E*), indicating high trapping efficiency ($k = 2.6 \times 10^8$ M^{-1} s^{-1}) (27). The characteristic DMPO/$^\bullet$SG adduct ESR spectrum is shown in Fig. 6. The DMPO/$^\bullet$SG adduct ESR spectrum was simulated using hyperfine coupling constants ($a^N = 15.2$ G and $a^H = 16.2$ G) similar to earlier reports (12, 13, 28, 29). The DMPO/$^\bullet$SG adduct spectrum was dependent on the presence of horseradish peroxidase, tyrosine, H_2O_2, GSH, and DMPO (data not shown). The addition of ascorbate resulted in a decrease in the DMPO/$^\bullet$SG adduct ESR intensity (Fig. 6, *spectra A–G*). Double integration of the low field transition (at 3459 G) of the DMPO/$^\bullet$SG adduct ESR spectrum is plotted *versus* ascorbate concentration in Fig. 7*B* (■). This decrease in the amount of DMPO/$^\bullet$SG adduct can be attributed to two mechanisms. The first mechanism is the preferential oxidation of the ascorbate (Equation 5), as opposed to GSH (Equation 1), by the tyrosyl radical, which would result in less GS$^\bullet$ formation and, hence, less DMPO/$^\bullet$SG adduct. The second mechanism is the reduction of the DMPO/$^\bullet$SG adduct by ascorbate to the corresponding ESR-silent hydroxylamine. The reduction of the DMPO/$^\bullet$SG by ascorbate complicates the interpretation of the DMPO/$^\bullet$SG adduct results.

In order to evaluate the contribution of the reduction of the DMPO/$^\bullet$SG adduct by ascorbate to the overall ascorbate-dependent decrease in DMPO/$^\bullet$SG adduct ESR intensity, the DMPO/$^\bullet$SG adduct was allowed to form (in the absence of ascorbate) for 90 s by the horseradish peroxidase system with 8 mM GSH. Immediately following the 90-s formation time, catalase was added to remove any remaining H_2O_2 and, hence, eliminate any further GS$^\bullet$ formation and, therefore, any additional

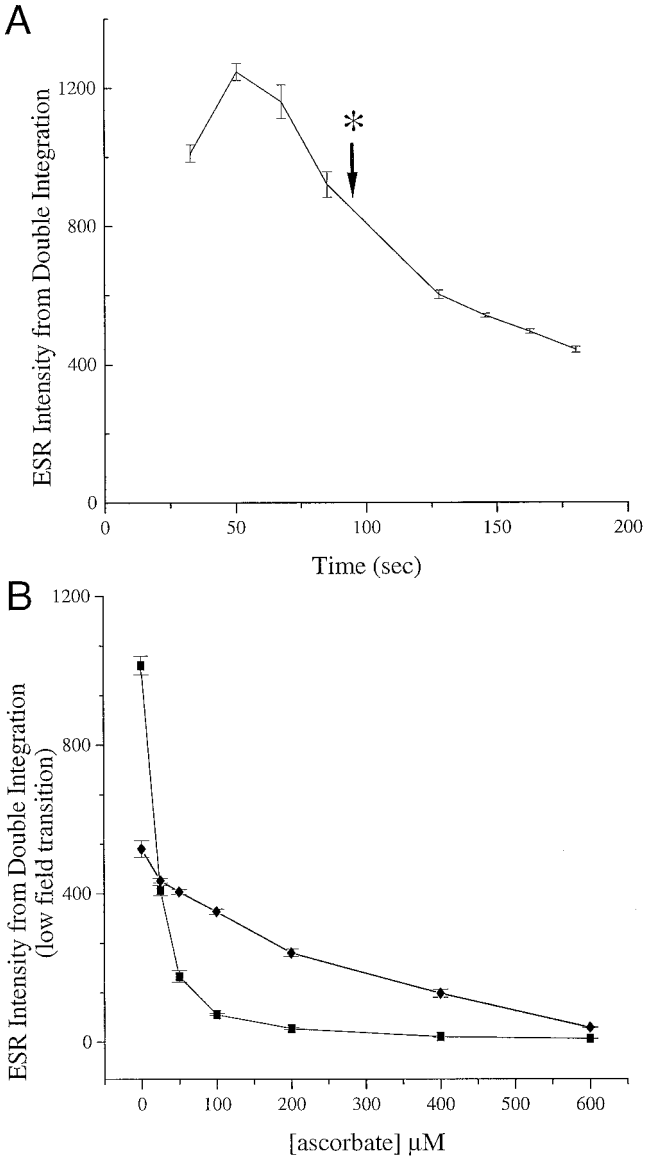
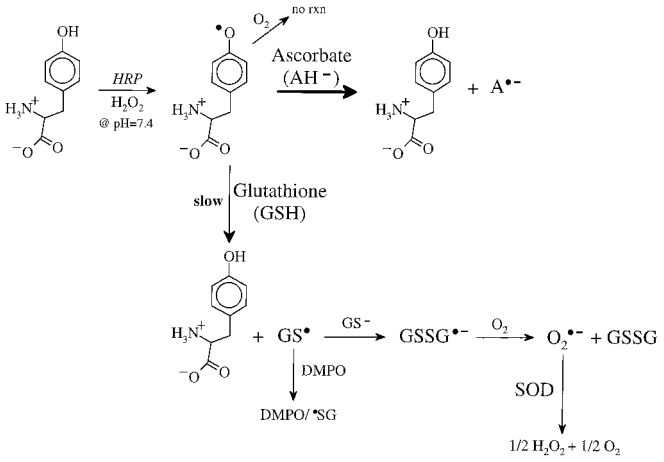


FIG. 7. **Ascorbate dependent decrease in the DMPO/˙SG adduct ESR intensity.** The intensity changes of the DMPO/˙SG adduct ESR spectrum were monitored as a function of time and ascorbate concentration to determine the relative contributions of the two mechanisms responsible for the decrease in the DMPO/˙SG adduct ESR spectrum, as described in the text. *A*, a plot of the ESR intensity from double integration shows the steady-state concentration of the DMPO/˙SG adduct ESR intensity as a function of time, generated under conditions identical to *spectrum A* in Fig. 6. The *asterisk* (*) indicates the time point of catalase/ascorbate addition as described in the text. *B*, (■) double integrated intensity of the low field ESR transition as a function of ascorbate concentration (pre-addition) measured ~33 s after tyrosyl radical generation, (♦) double integrated intensity of the low-field ESR transition as a function of ascorbate concentration (post-addition) measured ~128 s after tyrosyl radical generation. The *error bars* represent the S.E. for $n = 3$.

DMPO/˙SG adduct formation. Immediately following the addition of the catalase, varying amounts of ascorbate were added. This post-addition of ascorbate resulted in a decrease in the DMPO/˙SG radical adduct ESR spectrum due only to reduction of DMPO/˙SG adduct to the hydroxylamine form by ascorbate. The double integration of the low field transition (at 3459 G) of the DMPO/˙SG adduct ESR spectrum is plotted *versus* the post-addition ascorbate concentration in Fig. 7*B* (♦). Due to the decrease in the steady-state concentration of the radical adduct over time (Fig. 7*A*), the decay data in Fig. 7*B* are not directly comparable, but it can be seen that the rate of decay of the DMPO/˙SG adduct by the direct ascorbate (post-addition) reduction of DMPO/˙SG adduct (Fig. 7*B* (♦)) is significantly slower then the overall decrease observed (Fig. 7*B* (■)). While reduction of the DMPO/˙SG adduct by ascorbate does occur, this was not the dominant mechanism resulting in the decrease of the DMPO/˙SG adduct. It should be noted in Fig. 7*B* (■) DMPO/˙SG was being generated; where in Fig. 7*B* (♦), the DMPO/˙SG adduct was not being generated because the H_2O_2 has been eliminated from the system.



SCHEME 1

DISCUSSION

The results presented clearly demonstrate that the tyrosyl radical preferentially oxidizes ascorbate, as opposed to GSH. This conclusion is supported by: 1) nearly complete elimination of oxygen consumption upon the addition of 100 μM ascorbate to a complete oxygen-consuming horseradish peroxidase/H_2O_2/tyrosine system containing 8 mM GSH (Fig. 2, *D*); 2) only minor perturbation of the observed ascorbate radical ESR signal intensity when 8 mM GSH was added to a complete horseradish peroxidase/H_2O_2/tyrosine system with 100 μM ascorbate (Fig. 5, *B*); and 3) the near total elimination of the DMPO/˙SG adduct formation upon addition of 100 μM ascorbate to a complete horseradish peroxidase/H_2O_2/tyrosine system containing 8 mM GSH and 100 mM DMPO (Fig. 6, *D*).

The formation of the tyrosyl radical and the subsequent oxidation of ascorbate or GSH are outlined in Scheme 1. In all of the experiments, tyrosine stimulated ascorbate and GSH oxidation, demonstrating that the tyrosyl radical was the major radical product of the horseradish peroxidase compound I reaction, even in the presence of ascorbate or GSH. The radical sink hypothesis put forth by Winterbourn (1, 2) follows a reaction pathway initiated by the reaction of an oxidizing free radical with GSH. When tyrosine is the radical, this reaction does not occur in the presence of physiological concentrations of ascorbate, GSH, and O_2. Instead, the tyrosyl radical reacts directly with ascorbate to form the ascorbate radical and regenerate tyrosine.

In order to evaluate relative biological significance of ascorbate and GSH, one needs to consider physiological concentrations and mechanisms of regeneration. Intracellular levels of GSH in mammalian cells range from 0.5–10 mM (30–32) with many cells having 2–5 mM levels (32, 33). There is evidence that intracellular compartmentalization of GSH occurs (34–36). GSH levels in blood plasma are quite low (25 μM) (37). The regeneration of GSH is mainly controlled by glutathione disulfide reductase (30). The concentration of GSH used throughout these experiments represents the upper limit of the concentra-

tions found in mammalian tissue.

Ascorbate concentrations in human tissue are quite variable, ranging from roughly 40 μM in blood plasma (38, 39) to over 1 mM in the eye lens and the pituitary gland (38). Tissues such as the liver, kidneys, spleen, pancreas, lungs, heart, brain, and skin range between 100 and 800 μM (33, 38, 39). The concentration of ascorbate used in the present experiments represents the lower limit of the concentrations found in mammalian tissue. With respect to regeneration mechanisms of ascorbate, the ascorbate radical will undergo a pH-dependent second-order dismutation at the rate of approximately $2 \times 10^5\ M^{-1}\ s^{-1}$ at pH 7 (40), as well as enzymatic reduction by semidehydroascorbate reductase (26, 41). In addition, dehydroascorbate is reduced to ascorbate by an NAD(P)H-dependent enzymatic system (41).

The ascorbate/GSH concentrations and ratios used in this study realistically represent physiological concentrations. The superoxide pathway proposed by Winterbourn could occur in a biological system which does not have sufficient ascorbate such as cultured cells, which can be devoid of ascorbate (6). Even in systems devoid of ascorbate, GSH may not be properly labeled an antioxidant since other reactive radicals ($GS^{\bullet}$, $GSSG^{\bullet-}$, $GSOO^{\bullet}$, and $O_2^{\bullet-}$) are generated. Ascorbate is the superior reducing agent with a reduction potential of +0.3 V *versus* +0.9 V for GSH (3, 42). The ascorbate radical is a highly delocalized π radical and is quite unreactive, which makes it a highly desirable biological antioxidant. Unlike $GS^{\bullet}$, the ascorbate radical is not known to cause any biological damage (40).

These results clarify the roles of ascorbate and GSH in neutralizing the tyrosyl radical. The results are not specific for the tyrosyl radical and should hold true for many oxidizing free radicals formed by peroxidases (9–13).

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The Fate of the Oxidizing Tyrosyl Radical in the Presence of Glutathione and Ascorbate: IMPLICATIONS FOR THE RADICAL SINK HYPOTHESIS

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