Characterization of One- and Two-electron Oxidations of Glutathione Coupled with Lactoperoxidase and Thyroid Peroxidase Reactions*

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Glutathione (GSH) was oxidized to GSSG in the presence of H₂O₂, tyrosine, and peroxidase. During the GSH oxidation catalyzed by lactoperoxidase, O₂ was consumed and the formation of glutathione free radical was confirmed by ESR of its 5,5′-dimethyl-1-pyroline-N-oxide adduct. When lactoperoxidase was replaced by thyroid peroxidase in the reaction system, the consumption of O₂ and the formation of the free radical became negligibly small. These results led us to conclude that, in the presence of H₂O₂ and tyrosine, lactoperoxidase and thyroid peroxidase caused the one-electron and two-electron oxidations of GSH, respectively. It was assumed that GSH is oxidized by primary oxidation products of tyrosine, which are phenoxyl free radicals in lactoperoxidase reactions and phenoxylation cations in thyroid peroxidase reactions.

When tyrosine was replaced by diiodotyrosine or 2,6-dichlorophenol, the difference in the mechanism between lactoperoxidase and thyroid peroxidase disappeared and both caused the one-electron oxidation of GSH. Iodides also served as an effective mediator of GSH oxidation coupled with the peroxidase reactions. In this case the two peroxidases both caused the two-electron oxidation of GSH.

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Glutathione (GSH) plays a role in the protection of cellular constituents against oxidative damage and its redox property is an important subject studied by many workers. In general the oxidation of thiols can be divided formally into two classes; one-electron and two-electron reactions (1). In both cases the product appears to be the disulfide.

For one-electron oxidation,

\[ \text{RSH} \rightarrow \text{RS}^- + \text{H}^+ + e^- \]  

\[ 2 \text{RS}^- \rightarrow \text{RSSR} \]  

For two-electron oxidation,

\[ \text{RSH} \rightarrow \text{RS}^- + \text{H}^+ + 2e^- \]  

\[ \text{RSH} + \text{RS}^- \rightarrow \text{RSSR} + \text{H}^+ \]

The technique of pulse radiolysis has been successfully used for kinetic studies of thyl radical reactions (2-4). The involvement of thyl radicals has been suggested in the autoxidation of thiols (5, 6). Recently, the formation of thyl radicals has been confirmed by the spin-trapping ESR technique for the thiol oxidation reactions promoted by peroxidase and prostaglandin synthetase (7-11). Therefore, reactions of thyl radicals with O₂ could be physiologically important (5, 8, 12, 13).

GSH is oxidized in biological systems mostly by the glutathione peroxidase reaction that serves as a defense system against hydroperoxides (14-16). This GSH oxidation appears to occur by way of a two-electron transfer (1, 16). However, no proper experimental data have been presented for comparison of one-electron and two-electron oxidations of GSH. Careful consideration is needed in order to conclude that an oxidation-reduction reaction is a two-electron type (17). As reported previously (18), thyroid peroxidase selects either one-electron or two-electron oxidation mechanisms of phenols, depending on their substituents, while lactoperoxidase and horseradish peroxidase invariably catalyze one-electron oxidation of phenols. Since GSH was found to be oxidized by the primary oxidation products of phenols (18), we thought that these reaction systems might be used to characterize one-electron and two-electron oxidations of GSH.

**MATERIALS AND METHODS**

Thyroid peroxidase used in these experiments was purified from hog thyroid microsomes by immunosaffinity chromatography without the procedure of trypsin digestion (19). Lactoperoxidase was prepared from fresh bovine milk by the method of Morrison and Hultquist (20). The ratio of \( A_{390} \) to \( A_{380} \) was 0.24 for thyroid peroxidase and 0.92 for lactoperoxidase. The concentration of these peroxidases was calculated on the basis of a value of 114 for \( A_{390} \) at 413 nm (21).

GSH, GSSG, DMPO, bovine catalase, and superoxide dismutase were purchased from Sigma; L-tyrosine, monoiodotyrosine, diiodotyrosine, 5,5′-dithiobis-(2-nitrobenzoic acid), penicillamine, and di-thioceritrol from Nakarai (Kyoto); 2,6-dichlorophenol from Wako Pure Chemical Industry (Osaka); glutathione reductase from Oriental Co. (Osaka); and NADPH from Boehringer Mannheim.

The O₂ concentration was measured polarographically with a Clark-type O₂ electrode. The oxidation product of GSH was measured with a Hitachi 655 HPLC system equipped with a Merck LiChrosorb NH₂ column (22). The ESR spectra were recorded on a Varian E-109B spectrometer equipped with an aqueous flat cell. The reactions were carried out in 0.1 M potassium phosphate (pH 7.4) at 25 °C.

**RESULTS**

Lactoperoxidase and thyroid peroxidase both catalyze \( \text{H}_2\text{O}_2 \)-dependent oxidation of tyrosine to dityrosine, which can be measured from the increase of absorbance at 310 nm (23, 24). Although these peroxidases were unable to catalyze directly the oxidation of GSH, the formation of dityrosine was inhibited by GSH and the inhibition was removed after a certain lag time which depended on the amount of added

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1 The abbreviations used are: DMPO, 5,5′-dimethyl-1-pyrroline-N-oxide; HPLC, high pressure liquid chromatography.
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GSH (Fig. 1). Since the final oxidation product of tyrosine did not oxidize GSH, we concluded that GSH was oxidized by an intermediate oxidation product of tyrosine (18). The time course of tyrosine oxidation was monophasic for lactoperoxidase and diphasic for thyroid peroxidase (Fig. 1). The reason for this will be discussed later. No essential difference was seen in the inhibitory pattern of GSH for the two peroxidase reactions.

A remarkable difference was observed between the two reactions when the O2 consumption was measured in the presence of a large amount of GSH. As can be seen in Fig. 2A, a considerable amount of O2 was consumed when lactoperoxidase was added to a GSH solution containing H2O2 and tyrosine, but the O2 consumption became negligibly small when lactoperoxidase was replaced by thyroid peroxidase. This difference between the two peroxidases disappeared when tyrosine was replaced by 2,6-dichlorophenol (Fig. 2B). The O2 consumption appeared to occur invariably in the phenol-mediated oxidation of GSH catalyzed by lactoperoxidase, but it depended on phenol molecules when thyroid peroxidase was used as catalyst (Table I). The concentrations of the two peroxidases were chosen so as to give a similar rate of oxidation of a phenol. This classification of phenols was in accord with that determined from analysis of rate-determining intermediates of the enzyme during catalytic reactions (18). Table II shows that the molar ratio of GSSG formed per H2O2 was nearly 1.0 when H2O2 was added at concentrations below 80 μM in the tyrosine-mediated oxidation of GSH catalyzed by thyroid peroxidase. At higher concentrations of H2O2, catalytic decomposition of H2O2 became appreciable and the ratio of [GSSG]/[H2O2] was decreased. In the case of lactoperoxidase the 1:1 stoichiometry was observed only under anaerobic conditions (Table II). Under aerobic conditions GSH was oxidized at the expense of the tyrosine-mediated oxidation of GSH catalyzed by thyroid peroxidase. More details are described in the legends to Figs. 2 and 8. The O2 uptake was negligibly slow in the absence of enzyme (see Footnote b).

![Fig. 1](image1.png)

**FIG. 1.** The effect of GSH on the peroxidase-catalyzed oxidation of tyrosine. The concentrations were: 500 μM tyrosine, 100 μM H2O2, and 170 nM lactoperoxidase (A) or 82 nM thyroid peroxidase (B). The GSH concentration is indicated on the figure.

![Fig. 2](image2.png)

**FIG. 2.** The O2 consumption during GSH oxidation mediated by tyrosine or 2,6-dichlorophenol in peroxidase systems. A, 200 μM tyrosine, 20 μM H2O2, 1.0 mM GSH, and 170 nM lactoperoxidase (LPO) or 82 nM thyroid peroxidase (TPO). B, 40 μM 2,6-dichlorophenol, 20 μM H2O2, 1.0 mM GSH, and 80 nM lactoperoxidase or 82 nM thyroid peroxidase.

**TABLE I**

<table>
<thead>
<tr>
<th>Activity of phenols and iodide as a mediator for the O2-consuming oxidation of GSH catalyzed by peroxidases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
</tr>
<tr>
<td>Monoiodotyrosine</td>
</tr>
<tr>
<td>2,4-Dimethylphenol</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
</tr>
<tr>
<td>Diiodotyrosine</td>
</tr>
<tr>
<td>2,6-Dibromophenol</td>
</tr>
<tr>
<td>2,6-Dinitrophenol</td>
</tr>
<tr>
<td>Potassium iodide</td>
</tr>
</tbody>
</table>

This sign means that no significant O2 uptake was detected.

In this case a slow O2 uptake was seen in the absence of enzyme, but the addition of enzyme markedly promoted the reaction (see Fig. 2B).

**TABLE II**

Stoichiometry of the tyrosine-mediated oxidation of GSH catalyzed by lactoperoxidase and thyroid peroxidase

Aliquots (0.1 ml) of the reaction solutions were assayed after 30 min incubation under aerobic conditions unless otherwise noted. GSH was measured from the increase of absorbance at 412 nm upon the addition of 0.6 unit of glutathione reductase and 100-300 μM NADPH. Both measurements were carried out in a final volume of 2 ml.

<table>
<thead>
<tr>
<th>H2O2 added</th>
<th>μM</th>
<th>GSH oxidized</th>
<th>GSSG formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid peroxidase</td>
<td>10</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>70</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>152</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>190</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>240</td>
<td>130</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>20</td>
<td>530</td>
<td>255</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>41</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>175</td>
<td>91</td>
</tr>
</tbody>
</table>

1.1 mM GSH, 100 μM tyrosine, and 80 nM thyroid peroxidase.

1.1 mM GSH, 200 μM tyrosine, and 170 nM lactoperoxidase.
One-electron and Two-electron Oxidations of GSH

Table 1

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (mM)</td>
<td>GSSG (mM)</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

FIG. 3. Time courses of GSH disappearance and GSSG formation in the tyrosine-mediated oxidation of GSH catalyzed by lactoperoxidase. The concentrations were: 200 μM tyrosine, 20 μM H₂O₂, 1.1 mM GSH, and 170 nM lactoperoxidase. Aliquots (0.1 ml for GSH determination and 0.2 ml for GSSG determination) were withdrawn for assay at times indicated. GSH was measured by the addition of 5,5'-dithiobis-(2-nitrobenzoic acid) (0.2 mM) and GSSG was measured as described in Table I, both in a final volume of 2 ml.

FIG. 4. Assay of oxidation products of GSH by HPLC. The products were assayed 40 min after the reactions were started according to method of Wefers and Sies (12). The positions indicated represent: 1, N-ethylsuccinimidyl GSH; 2, glutathione sulfonate; and 3, GSSG. The others are attributed to impurities contained in the GSH and GSSG preparations used. A, a mixture of 1.1 mM GSH and 0.25 mM GSSG (control); B, 2.2 mM GSH, 1.0 mM xanthine, and 200 nM xanthine oxidase under O₂-saturated conditions. C, 1.1 mM GSH, 200 μM tyrosine, 20 μM H₂O₂, and 170 nM lactoperoxidase under aerobic conditions.

A very small amount of glutathione free radical was formed in the reaction of thyroid peroxidase. This fact appeared to correspond with the previous finding (18) that the formation of a small amount of benzoquinone mostly to benzoquinone. DMPO inhibited the tyrosine-mediated O₂-consuming oxidation of GSH catalyzed by lactoperoxidase. The concentration of DMPO to give 50% inhibition was 18 mM.

The tyrosine-mediated O₂-consuming oxidation was not specific for GSH; other sulfhydryl compounds such as cysteine, penicillamine, and dithioerythritol were also oxidized by O₂ in the presence of H₂O₂, tyrosine, and lactoperoxidase (Fig. 6). H₂O₂ was necessary for the reaction to proceed, but in some cases it was not necessary to be added to the reaction solution, particularly when 2,6-dichlorophenol served as the mediator (Fig. 7). In a solution containing GSH and 2,6-dichlorophenol, the O₂ consumption occurred slowly in the absence of lactoperoxidase probably because 2,6-dichlorophenol was autoxidized to produce its free radical and superoxide, which caused chain reactions of GSH oxidation. The O₂ uptake burst upon the addition of the enzyme and was inhibited by catalase and superoxide dismutase. The mechanism will be discussed later. It has been reported (26) that the aerobic oxidation of dithioerythritol is catalyzed by peroxidases in the absence of mediator. In our cases the reaction was negligibly slow without a mediator.

We have reported (27) that the oxidation of iodide is very fast in the reactions of lactoperoxidase and thyroid peroxidase and also that GSH is a good acceptor for the enzyme-bound oxidized iodide. Fig. 8 shows that a small amount of iodide greatly accelerated the oxidation of GSH. In this reaction, the iodide acted as the mediator and only GSSG was detected as the oxidation product of GSH by means of HPLC. No O₂ consumption was observed in the iodide-mediated oxidation...
systems.
without KI and with lactoperoxidase or thyroid peroxidase.
small.
of iodide and iodide
PM
HzO₂ and GSH were measured from the kinetic analysis of the effects
of GSH even when the reaction was caused either by lactoperoxidase or thyroid peroxidase under aerobic conditions. H₂O₂ itself was reported to be oxidized by the oxidized iodide (28-30) and the rate of the reaction of H₂O₂ with the oxidized iodide was measured kinetically from the effect of H₂O₂ concentration upon the rate of O₂ evolution at varied iodide concentrations. The rate constant was compared with that for GSH in Table III, which showed that the oxidized iodide reacted mostly with GSH rather than H₂O₂ under the present experimental conditions.

FIG. 7. The effect of catalase and superoxide dismutase (SOD) on the aerobic oxidation of GSH in the presence of 2,6-dichlorophenol and lactoperoxidase. The concentrations were: 1.1 mM GSH, 50 μM dichlorophenol, and 170 nM lactoperoxidase. The concentrations of catalase and superoxide dismutase were indicated on the figure.

FIG. 8. Iodide-mediated oxidation of GSH in peroxidase systems. The concentrations were: 1.05 mM GSH, 95 μM H₂O₂, 2.5 μM KI (Δ, C) or 7.5 μM KI (Δ, A) and 65 nM lactoperoxidase (Δ, A) or 22 nM thyroid peroxidase (○, O). The chain line denotes control without KI and with lactoperoxidase or thyroid peroxidase.

TABLE III
Rate constants for reactions of GSH and H₂O₂ with Compound I (EO) and the assumed enzyme-bound iodinium cation (EO⁺I⁺) of lactoperoxidase and thyroid peroxidase

<table>
<thead>
<tr>
<th>System</th>
<th>Reaction</th>
<th>Rate Constant (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactoperoxidase</td>
<td>GSH</td>
<td>3.6 × 10⁶</td>
</tr>
<tr>
<td>Thyroid peroxidase</td>
<td>GSH</td>
<td>4.8 × 10⁷</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>2.1 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>4.0 × 10⁹</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>5.3 × 10⁸</td>
</tr>
<tr>
<td></td>
<td>H₂O₂</td>
<td>1.1 × 10⁸</td>
</tr>
</tbody>
</table>

*This sign means that catalytic oxidation of GSH was negligibly small.


discussion
From the spectrophotometric analysis of catalytic intermediates of thyroid peroxidase, we have concluded (18) that thyroid peroxidase catalyzes two-electron oxidation of some phenolic molecules and one-electron oxidation of the other. This classification is in good accord with that listed in Table I. These results will be explained as follows. Here, we tentatively use chemical formula of φ-O⁻ and GS⁺ for two-electron oxidation products of phenols and GSH, respectively, to simplify the discussion. The oxidation of phenols (φ-OH) catalyzed by peroxidases will be; for one-electron oxidation,

\[
2 \phi-OH + H_2O_2 \xrightarrow{\text{peroxidase}} 2 \phi-O^- + 2 H_2O
\]  

and for two-electron oxidation,

\[
\phi-OH + H_2O_2 \xrightarrow{\text{peroxidase}} \phi-O^+ + 2 H_2O
\]  

GSH seems to be oxidized by these primary oxidation products of phenols as follows.

\[
GSH + \phi-O^- \rightarrow GS^- + \phi-OH
\]  

\[
GSH + \phi-O^+ \rightarrow GS^+ + \phi-OH
\]  

Finally, GS⁻ and GS⁺ are both converted to GSSG through Reactions 2 or 4. This implies that there is no apparent difference in the final products between the above two reactions under anaerobic conditions. Little experimental evidence has been reported on the existence of GS⁺ as an intermediate of GSH oxidation. It is reasonable to assume that no reaction occurs between GS⁻ and O₂. On the other hand, special attention has been devoted to the reactivity of GS⁻ particularly under aerobic conditions (10, 12, 13, 31) and the following reactions have been presented.

\[
GS^- + GSH = GSSG^- + H^+
\]  

\[
GSSG^- + O_2 \rightarrow GSSG + O_2^-
\]  

\[
GS^- + O_2 \rightarrow GSOO^-
\]  

The rate constant for GSSG⁻ formation (Reaction 9) has been reported to be 6.2 × 10⁻⁴ M⁻¹ s⁻¹ (2). A final product of Reaction 11 might be glutathione sulfonate as reported by Wefers and Sies (12), who have found that GSH is oxidized to a mixture of GSSG with 6-10% glutathione sulfonate when GSH is present in the xanthine oxidase reaction. We also confirmed their results under similar conditions but could not detect the formation of glutathione sulfonate in the tyrosine-mediated O₂-consuming oxidation of GSH catalyzed by lactoperoxidase. This difference is not yet explained.

We assume that tyrosine is oxidized by the thyroid peroxidase system through Reaction 6. However, there is some complication in the two-electron oxidation catalyzed by thyroid peroxidase. Even though tyrosine reduces Compound I compulsorily to the ferric state, the one-electron reduction of Compound I to Compound II will take place slowly at the expense of unidentified endogenous electron donors (32). This one-electron reduction of Compound I will result in the formation of a small amount of glutathione free radical as seen in Fig. 5. Since Compound II of thyroid peroxidase is much less reactive toward tyrosine and iodide (18), its accumulation will slow down the oxidation of tyrosine (Fig. 1B).

The peroxidase-catalyzed aerobic oxidation of GSH appears to occur through a free-radical chain reaction consisting of Reactions 9, 10, and 12.

\[
GSH + O_2^- + H^+ \rightarrow GS^- + H_2O_2
\]  

Reaction 12 has been reported (12, 33) and is a key reaction to be involved in most peroxidase-catalyzed aerobic oxidations.
Iodide is a good electron donor in the reactions of lactoperoxidase and thyroid peroxidase, and is oxidized by Compound I (EO) of these peroxidases to an iodinium cation, which is assumed to be present as an enzyme-bound form (29, 35).

\[ \text{EO} + \Gamma + \rightarrow \text{EO}^+\Gamma^- \]  

This iodinium cation reacts with various reductants (27). When GSH and H\(_2\)O\(_2\) are used as the reductant, the reactions will be the following.

\[ \text{EO}^+\Gamma^- + \text{GSH} + \text{H}^+ \rightarrow \text{E} + \text{H}_2\text{O} + \text{GSI} \]  

\[ \text{GSI} + \text{GSH} \rightarrow \text{GSSG} + \Gamma^- + \text{H}^+ \]  

\[ \text{EO}^+\Gamma^- + \text{H}_2\text{O}_2 \rightarrow \text{E} + \text{O}_2 + \Gamma^- + \text{H}_2\text{O} \]  

Through these reactions the iodide may serve as an effective mediator in the peroxidase-catalyzed oxidation of GSH (36) and decomposition of H\(_2\)O\(_2\) (29). These reactions are concluded to occur by way of two-electron transfer. The marked difference between GSH and H\(_2\)O\(_2\) is that Compound I of lactoperoxidase and thyroid peroxidase reacts directly with GSH even under aerobic conditions. In this sense this reaction system is similar to that of selenium glutathione peroxidase.

After completion of this work, a publication appeared from Harman et al. (37), who reported one- and two-electron oxidation of GSH by peroxidases. They concluded that horseradish peroxidase and glutathione peroxidase catalyze one- and two-electron oxidation of GSH, respectively. The techniques used to classify the two mechanisms in this work are essentially the same as those reported by them. Since GSH is a very slow substrate for heme-containing peroxidases, we used phenols or iodide as a mediator in the peroxidase-catalyzed oxidation of GSH and demonstrated clear differences in the mechanisms of GSH oxidation between lactoperoxidase and thyroid peroxidase.

Acknowledgment—We wish to thank Dr. Tomio Kotani for providing a monoclonal antibody of thyroid peroxidase.

REFERENCES