Oxygen Activation by Sulfhydryl Oxidase and the Enzyme’s Interaction with Peroxidase

HAROLD E. SWAISGOOD and PHILIP ABRAHAM
Department of Food Science
North Carolina State University
Raleigh 27650

ABSTRACT

Sulfhydryl oxidase is a metalloglycoprotein in milk which catalyzes oxidation of thiols to their corresponding disulfides using molecular oxygen as an electron acceptor. Cysteine, peptides, and proteins all serve as substrates for this oxidative activity. Investigation of the various possible active oxygen species suggests that the enzyme-bound forms of singlet oxygen and a hydroperoxy group may be produced during catalysis. However, the possible intermediate superoxide anions or hydroxyl radicals did not appear to be formed. Evidence was obtained for a direct interaction between sulfhydryl oxidase and horseradish peroxidase which results in an enhancement of the thiol oxidative activity. This interaction also induced a change in the peroxidase absorption spectra consistent with formation of the horseradish peroxidase-II form of the enzyme. Stimulation of oxidase activity also was observed in the presence of oxytocin and certain concentrations of oxidized glutathione.

INTRODUCTION

In recent years a number of investigations of enzymes which catalyze thiol-protein-disulfide interchange have been reported (7). Some of these enzymes catalyze the oxidation of sulfhydryl groups in proteins using small peptide disulfides as electron acceptors. Others catalyze the reduction of disulfides in proteins in the presence of small thiols such as reduced glutathione (GSH).

In aerobic biology, however, the ultimate electron sink is molecular oxygen. Hence, it is not surprising to find enzymes capable of catalyzing oxidation of thiols using this element as the electron acceptor. Moreover, it has become apparent in recent years that partially reduced species of oxygen, intermediate between molecular oxygen and water, and also singlet oxygen are formed during biological oxidations and may exist at finite concentrations during both autoxidation reactions and enzyme-catalyzed reactions (5, 10, 17, 18, 19). Singlet oxygen, for example, has been implicated in the enzymatic and nonenzymatic (light- and copper-catalyzed) peroxidation of milk lipids (4). Some of these intermediate species, especially the hydroxyl radical, are powerful oxidants. Consequently, it is necessary to understand mechanisms by which such intermediates are generated and consumed to understand and possibly control factors related to flavor and nutrition in foods.

For example, autoxidation of thiols can occur in the presence of oxygen and trace amounts of transition metal ions. Recently the formation and participation of both superoxide and hydroxyl radicals in this autoxidation reaction has been demonstrated (18). However, most enzyme catalyzed oxidations do not initiate destructive radical reactions (19).

In this paper we shall describe some of the characteristics of the enzyme sulfhydryl oxidase which catalyzes the oxidation of thiol groups using molecular oxygen directly as the electron acceptor.

GENERAL CHARACTERISTICS OF SULFHYDRYL OXIDASE

This enzyme is a metalloglycoprotein with an iron prosthetic group which is required for...
activity (14, 21). The subunit molecular weight as determined by gel electrophoresis in SDS or light scattering in guanidinium chloride is approximately 89,000 (12, 14, 16). However, in whey the enzyme exists as highly aggregated particles of varying size, the larger particles approaching 300 nm in diameter (12, 15, 16).

In comparison with other enzymes which catalyze the formation of disulfide bonds using other disulfides as electron acceptors, sulfhydryl oxidase appears to be unique in that it catalyzes aerobic oxidation of sulfhydryl groups generating disulfide bonds de novo. The stoichiometry of the reaction catalyzed has been established as (14):

\[
2 \text{RSH} + \text{O}_2 \rightarrow \text{RSSR} + \text{H}_2\text{O}_2
\]

In addition to oxidation of sulfhydryl groups in reduced proteins (13, 21), a number of small thiols are also very good substrates. In fact, judging from the Michaelis constant, GSH is the best substrate \( (K_m \approx 0.3 \text{ mM}) \) and is used routinely to assay this enzyme (14, 21). Generally speaking, amino acids and peptides are the best substrates and other thiols (for example, mercapto acids) are oxidized only slowly if at all.

### Table 1. Effect of potential inhibitors on sulfhydryl oxidase activity.\(^a\)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Phenylenediamine</td>
<td>36</td>
</tr>
<tr>
<td>o-Dianisidine</td>
<td>55</td>
</tr>
<tr>
<td>Diazabicyclooctane</td>
<td>18</td>
</tr>
<tr>
<td>Guanine</td>
<td>30</td>
</tr>
<tr>
<td>Dioxane</td>
<td>0</td>
</tr>
<tr>
<td>Catechol</td>
<td>0</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoic acid</td>
<td>0</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)Assay mixtures contained .8 mM GSH at pH 7.0. The inhibitor concentrations ranged from about .6 to 3 mM.

o-phenylenediamine and o-dianisidine or in the presence of singlet oxygen trappers guanine and diazabicyclooctane. Inhibition by the peroxidase substrates can be rationalized by suggesting the formation of an enzyme-bound hydroperoxy group. The reaction of these substrates with a similar horseradish peroxidase complex has been proposed (9).

### Intermediate Oxygen Species

#### Studies of Potential Inhibitors

A number of compounds have been tested for their effect on sulfhydryl oxidase-catalyzed oxidation of GSH with the intention of identifying possible reduced oxygen intermediates in the reaction (1). Results summarized in Table 1 show that many of these compounds do not exhibit any inhibition. Thus, mannitol and ethanol, excellent hydroxyl radical trappers, do not inhibit the enzyme. Also, judging from the lack of inhibition when superoxide dismutase was added, superoxide anion is not formed as an intermediate. Moreover, oxidation of nitrobluetetrazolium chloride was not observed during enzyme catalysis. Sulfhydryl oxidase activity was distinct from that of thiol oxidase (EC 1.8.3.2) by the inability of thiol oxidase substrates catechol and 3,4-dihydroxybenzoic acid to inhibit GSH oxidative activity.

However, inhibition was significant upon addition of horseradish peroxidase substrates Oxidation of 1,3-Diphenylisobenzofuran

To investigate further the possible existence of a singlet oxygen intermediate, the effect of the enzyme on 1,3-diphenylisobenzofuran, which is oxidized specifically by singlet oxygen to dibenzylibenzene (20), was examined. As illustrated by the progress curves in Figure 1, this compound is oxidized by the enzyme. When sulfhydryl oxidative activity was inactivated by heating the enzyme preparation, all furan oxidative activity was lost. Furthermore, the furan oxidative activity was inhibited by both inhibitors and substrates (presumably by competition) for the sulfhydryl oxidative activity of the enzyme. Hence, we conclude that in its active form, sulfhydryl oxidase is capable of activating molecular oxygen to the singlet state.

### Postulated Enzyme Mechanism

These observations, together with other data, permit the proposal of a tentative reaction scheme. According to this scheme, a mixed
Figure 1. Oxidation of 1,3-diphenylisobenzofuran (DPBF) by sulfhydryl oxidase. A 3.0-ml reaction mixture contained 0.13 mg enzyme (Fraction V) and 0.097 μmol DPBF. Curve 1: DPBF; the solution was made to 0.8 mM in GSH at the point indicated. Curve 2: DPBF plus 0.21 mM GSH. Curve 3: DPBF plus 0.23 mM KCN. Curve 4: DPBF with heat-inactivated enzyme. Curve 5: DPBF plus 0.2 mM O-dianisidine. The absorbance scale for heat-inactivated enzyme was adjusted to compensate for the turbidity. (Taken from Swaisgood and Horton (21)).

Evidence for the existence of enzyme-bound singlet oxygen and a hydroperoxy group was discussed and will be commented on later. Formation of an intermediate enzyme-substrate mixed-disulfide was suggested by specific binding of the enzyme to immobilized reduced glutathione and its release by thiol addition. The order of substrate binding and product release has not been investigated and, hence, is only speculative.

INTERACTION WITH HORSERadISH Peroxidase

Enhancement of Sulfhydryl Oxidase Activity

The rate of oxidation of GSH was increased by as much as 5-fold when horseradish peroxidase (HRP) was added to the assay. The rate of enhancement was proportional to the activity of HRP added and not to total protein concentration. Furthermore, the enhancement was not affected by addition of excess catalase; hence, it appears that some sort of direct interaction between the two enzymes, sulfhydryl oxidase and HRP, must occur.

Changes in the Horseradish Peroxidase Absorption Spectra

Further evidence for a direct interaction between these two enzymes also is provided by the effect of sulfhydryl oxidase-catalyzed GSH oxidation on the absorption spectra of HRP.

Figure 2. Postulated reaction scheme for sulfhydryl oxidase-catalyzed thiol oxidation. (Taken from Swaisgood and Horton (21)).
Upon initiation of the sulfhydryl oxidase-catalyzed reaction, absorption increased centered at roughly 417 nm as illustrated by the difference spectrum in Figure 3. The magnitude of the difference spectrum decreased with time, falling to zero when the substrate was exhausted. The position of the absorption maximum suggests that the species formed corresponds to HRP-II, which is the one-electron reduced form of oxidized HRP (3, 6, 8, 11). These observations are consistent with a direct transfer of an intermediate species of oxygen, presumably a hydroperoxy group, from sulfhydryl oxidase to the active site of HRP.

Postulated Mechanism for Horseradish Peroxidase Stimulation of Sulfhydryl Oxidase

In addition to excess catalase not affecting the stimulation of GSH oxidation by addition of peroxidase to sulfhydryl oxidase, GSH is a relatively poor substrate for HRP. Consequently, the rate enhancement could not be attributed to simple HRP-catalyzed GSH oxidation. Possibly HRP-II, formed by direct interaction with sulfhydryl oxidase, is capable of an increased rate of catalysis of GSH oxidation. Rate enhancement also could occur if protonation and dissociation of the hydroperoxy group in sulfhydryl oxidase were limiting. Abstraction of the hydroperoxy group through interaction of sulfhydryl oxidase with HRP could increase the rate of this step. In this regard, HRP binds its substrate in the reactive form HO₂ (2). Hence, the abstraction of a similar species directly from sulfhydryl oxidase presents a tenable hypothesis. These possible mechanistic interactions are summarized schematically in Figure 4.

INTERACTION WITH PEPTIDE DISULFIDES

Sulfhydryl oxidase activity also increased upon addition of the peptide disulfides GSSG and oxytocin to assay mixtures. Substrate inhibition occurs at concentrations of GSH greater than about 1 mM (14, 21). These results...
were interpreted as indicative of substrate binding to a second site on the enzyme. However, in the presence of .8 mM GSSG, for example, inhibition by excess GSH was not observed. Consequently, the increased activity of the enzyme in the presence of these disulfides may result from competitive binding at an allosteric binding site for substrate.

A subtle structural change in the enzyme resulting from the binding of these disulfides also was evidenced by changes in the enzyme's tryptophyl fluorescence spectrum. Fluorescence quenching increased with increasing concentrations of the disulfides. Oxytocin was 15 times more effective than GSSG both in stimulation of enzymatic activity and in fluorescence quenching. Whether such interaction bears physiological significance remains to be determined.

CONCLUSIONS

Sulfhydryl oxidase in milk and dairy products may have important consequences with regard to flavor and oxidative stability. It is in skim milk at a concentration of 5 to 10 mg/liter and is inactivated only partially by standard pasteurization. Consequently, cooked flavors do not persist in pasteurized milk although odors near a pasteurizer certainly suggest the formation of mercaptans. Enzymatic oxidation of sulfhydryl groups may result in a retardation of subsequent lipid oxidation in milk since strong oxidants such as hydroxyl radical and superoxide are not intermediates in the enzyme-catalyzed reaction. In contrast, these oxidants are formed during nonenzymatic autoxidation processes. An enzyme-bound singlet oxygen species appears to be formed; however, it does not appear to be released from the enzyme, and its accessibility for general oxidative reactions remains to be determined.

An interaction between sulfhydryl oxidase and horseradish peroxidase has been demonstrated which may involve a direct abstraction of a hydroperoxy group by the peroxidase. Obviously, a similar interaction with lactoperoxidase could have considerable importance, but this possibility remains for future investigation.

REFERENCES


