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THE OXIDATION OF DITHIOTHREITOL BY PEROXIDASES AND OXYGEN

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Summary

Horseradish peroxidase (1.11.1.7), lactoperoxidase (1.11.1.7), and the fragment of cytochrome *c* known as microperoxidase have been shown to catalyze the oxidation of reduced dithiothreitol in an oxygen-consuming reaction. Evidence for horseradish peroxidase intermediates compound III and compound II has been observed, although ferroperoxidase was not identified during the course of the reaction. The stoichiometry has been established as 1 : 1 for oxygen consumed to dithiothreitol oxidized. Cysteine and glutathione have also been shown to be substrates for horseradish peroxidase oxidase reaction.

Introduction

Several enzymes which catalyze the oxidation of thiols utilizing molecular oxygen have been reported. Sulfhydryl oxidases have been isolated from bovine milk [1] from *Piricularia oryzae* [2] and from *Myrothecium verrucaria* [3]. Recently a sulfhydryl oxidase has also been isolated from the male reproductive tract [4] which may be involved in the protection of sperm. The specificity of these oxidases varies.

In 1946 Randall [5] first noted that thiol compounds were substrates for horseradish peroxidase, however, only the peroxide-consuming phase of the reaction was observed. It was demonstrated that thiol compounds such as dithiouracil and thiourea are oxidized by hydrogen peroxide and that the rate of oxidation was accelerated by peroxidase. Since the first report of thiols catalyzed by peroxidases very little has been done in this area.

In our studies on the peroxidase activation of galactose oxidase we obtained evidence that repair of disulfide bonds by peroxidase may account for the acti-

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vation of galactose oxidase (Olsen, J. and Davis, L., unpublished). Therefore we undertook an investigation of a model system to discover if peroxidases are indeed able to catalyze the formation of disulfide bonds from thiols.

In this communication we report studies conducted on the ability of peroxidases to enzymatically catalyze oxidation by O_2 of dithiothreitol, cysteine and glutathione to their disulfides.

Materials

Horseradish peroxidase type II (1.11.1.7), catalase (1.11.1.6), microperoxidase (heme-containing portion of cytochrome *c*, of molecular weight 2000), lactoperoxidase (1.11.1.7), dithiothreitol, L-cysteine and reduced glutathione were purchased from Sigma Chemical Company.

Methods

Oxygen consumption was determined using a Yellow Springs Oxygen Monitor with Clark Electrode.

Kinetic studies were done using a Beckman Model 24 Spectrophotometer equipped with recorder and variable temperature block.

Spectra were taken on an Aminco DW-2-UV-VIS Spectrophotometer. Samples used to obtain low temperature spectra were prepared in phosphate buffer and frozen in a precooled sample chamber. Reaction mixtures were mixed and frozen within thirty seconds.

All solutions and samples for the anaerobic experiments were prepared with degassed, nitrogen or carbon monoxide saturated buffer, under nitrogen in a glove bag.

The purity of horseradish peroxidase was calculated from the absorbance at 403 nm, using ϵ (403 nm) = 100 $cm^{-1} \cdot mM^{-1}$ [6]. The concentration of oxidized dithiothreitol was calculated from its ϵ (283 nm) of 273 $cm^{-1} \cdot M^{-1}$ [7].

Results

The oxidation of dithiothreitol by horseradish peroxidase was observed spectrophotometrically at 283 nm, the λ_{max} for oxidized dithiothreitol [7]. The oxygen consumption of the horseradish peroxidase reaction was followed using the oxygen monitor. Fig. 1 shows a comparison of the reaction monitored by both methods under identical conditions. Each system was equilibrated at 31.5°C to air, and the spectrophotometric assay was performed in septum-capped cuvettes sealed under nitrogen to simulate the closed conditions found in the oxygen monitor assay. In Fig. 1 the recorder traces have been recalibrated to show μmol of dithiothreitol oxidized and μmol oxygen consumed. In the initial stage of the reaction the ratio of dithiothreitol oxidized to oxygen consumed is 1 : 1. A second phase of the reaction is observed in the spectrophotometric assay. It is known that peroxidase catalyzes the oxidation of thiols using hydrogen peroxide [5]. An enzymatic reaction between dithiothreitol, hydrogen peroxide, and horseradish peroxidase in the absence of oxygen produces oxidized dithiothreitol. When catalase is added to the system

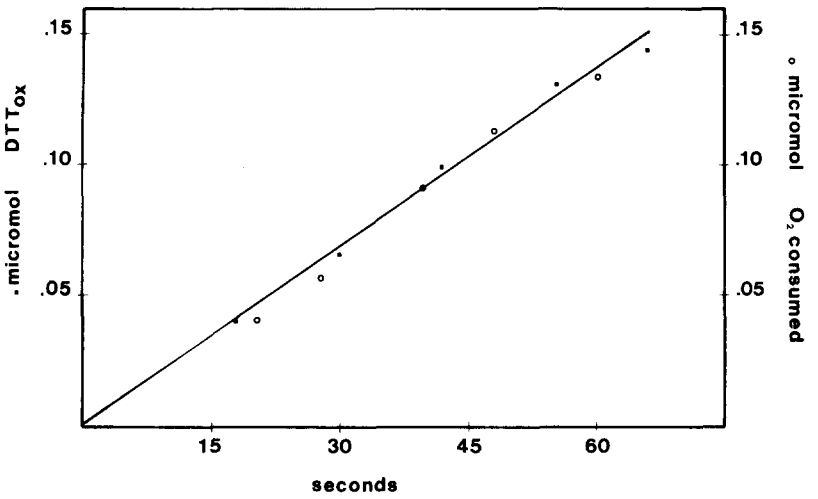


Fig. 1. The reaction was performed in 3 ml of 5 mM dithiothreitol prepared with 0.02 M pH 7.0 phosphate buffer equilibrated to air at 31.5°C. The reaction was initiated with the addition of 1.5 μM horseradish peroxidase. Solid points represent μmol of dithiothreitol oxidized at given times, and the open points represent μmol of oxygen consumed at given times. See text. DTT, dithiothreitol.

the peroxide-consuming oxidation of dithiothreitol is eliminated. The addition of catalase to the reaction mixture prior to addition of horseradish peroxidase does not produce a lag period.

Peroxidase showed a linear rate response to increasing substrate, and we were unable to saturate the enzyme with dithiothreitol. Peroxidase is also capable of catalyzing the oxidation L-cysteine and reduced glutathione in an oxygen-consuming reaction.

Lactoperoxidase and microperoxidase (a heme-containing fragment of cytochrome *c* which demonstrates peroxidase activity) were tested, to determine if disulfide formation was an oxidase reaction of other peroxidases. Both showed catalytic activity in the formation of disulfide bonds in an oxygen-consuming reaction.

Horeradish peroxidase intermediates were determined by spectral observations of reaction mixtures over the range of 350 to 700 nm. The 403-nm band of ferriperoxidase shifts to 416 nm during the reaction. The visible spectrum of horseradish peroxidase in the presence and absence of dithiothreitol at −77°C is shown in Fig. 2. The reaction mixture shows the appearance of new peaks at 543 nm and 579 nm and the disappearance of the 487 nm and 637 nm peaks of ferriperoxidase. The change in absorbance at 520 nm and 580 nm at room temperature as a function of time is presented in Fig. 3. The 520-nm absorbance shows an initial increase and then a steady decrease. Correspondingly the 580-nm peak shows an increase in absorbance. The 520-nm peak is indicative of formation of compound II and the 580-nm peak represents formation of compound III.

Direct reduction of ferriperoxidase to ferroperoxidase by dithiothreitol under anaerobic conditions was not observed. The same experiment done in the

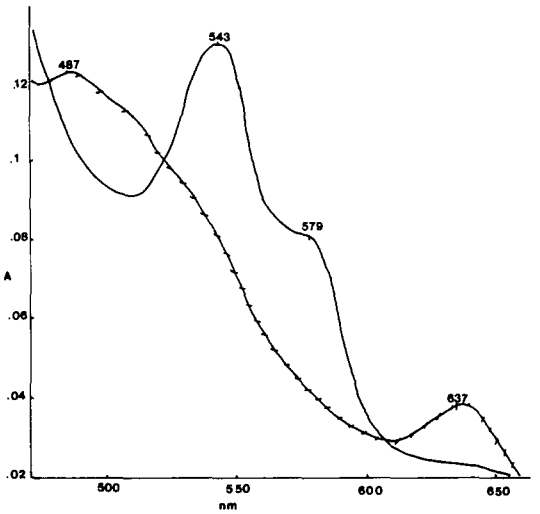
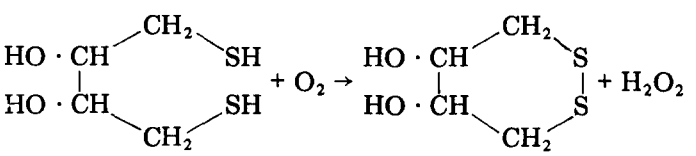


Fig. 2. Spectra of the horseradish peroxidase-dithiothreitol reaction taken in 0.1 M pH 7.0 phosphate buffer at −77° C. ——— 16 μM horseradish peroxidase, *——* 18 μM horseradish peroxidase and 2 mM dithiothreitol mixed just prior to freezing.

presence of carbon monoxide gave no carbon monoxide-ferroperoxidase. Ferroperoxidase could, however, be generated anaerobically by addition of solid dithionite. The carbon monoxide-ferroperoxidase was prepared by reduction of ferriperoxidase with solid sodium dithionite in the presence of carbon monoxide.

Discussion

The reaction of peroxidase on dithiothreitol appears to have two phases. The first more rapid reaction consumes oxygen and produces peroxide (Eqn. 1). An oxygen-consuming reaction:



(1)

also occurs with cysteine and reduced glutathione. This is similar to the initial reaction of horseradish peroxidase on dihydroxyfumaric acid [8].

Lactoperoxidase and microperoxidase will also catalyze the oxidation of dithiothreitol, so the reaction is not specific for horseradish peroxidase.

The ratio of dithiothreitol oxidized to oxygen consumed is 1 : 1 in the initial stages of the reaction where Eqn. 1 is of major importance. This is comparable

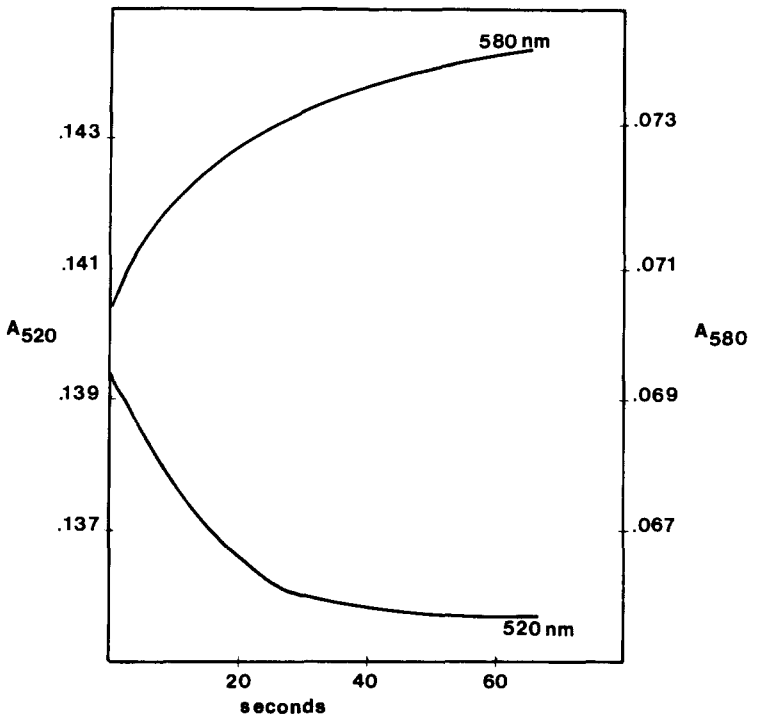


Fig. 3. Horseradish peroxidase-dithiothreitol reaction monitored at 520 nm and 580 nm. The reaction mixture contained 3 μM in horseradish peroxidase and 1 mM in dithiothreitol in 0.1 M pH 7.0 phosphate buffer at room temperature.

to the 1 : 1 ratio of oxygen consumed to diketosuccinate formed in the oxidase reaction of horseradish peroxidase with dihydroxyfumaric acid [9]. In later stages of the reaction the peroxide produced influences the ratio of dithiothreitol oxidized to oxygen consumed.

The presence of catalase in the reaction mixture prior to the addition of peroxidase does not result in a lag period for the uptake of oxygen in the peroxidase-dithiothreitol oxidase reaction, nor does the reaction show catalase inhibition. The indole acetic acid-horseradish peroxidase oxidase reaction is also reported not to require peroxide nor show catalase inhibition [6]. However, the indole acetic acid-peroxidase reaction unlike the dithiothreitol-peroxidase reaction exhibits a lag period in the absence of peroxide.

In this communication we have investigated the spectral shifts of horseradish peroxidase in the presence of dithiothreitol. Evidence has been obtained for formation of both compound II and compound III during the course of the reaction. Evidence for formation of compound II was obtained by monitoring the reaction mixture at 520 nm. One observes initially a rapid increase in absorbance at 520 nm followed by a decrease as shown in Fig. 3, suggesting formation and disappearance of compound II. Evidence for formation of compound III was obtained by recording the spectrum of the reaction mixture at $-77°C$ (Fig. 2) after 30 s and by monitoring the reaction mixture at room temperature at 580 nm. The absorbance at 580 nm increases with time (Fig. 3) as that at

520 nm decreases. Thus the enzymic oxidation of dithiothreitol by horseradish peroxidase appears to proceed through compound II to compound III. This sequence of intermediates has also been demonstrated for the action of horseradish peroxidase on dihydroxyfumarate [10]. No evidence for formation of ferroperoxidase could be obtained in this system and we could not demonstrate direct reduction of horseradish peroxidase by dithiothreitol under anaerobic conditions.

The ability of horseradish peroxidase to form disulfides in the presence of molecular oxygen represents a new and important reaction of peroxidases especially in the light of our observation that it will catalyze the conversion of the enzyme galactose oxidase from a sulfhydryl (inactive) to a disulfide (active) form (Olsen, J. and Davis, L., unpublished). The same reaction may occur in other reduced (SH) to oxidized (S-S) enzyme and protein transformations.

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