Note

The Reactivity of 4-Thiouridine with Peroxidase and Superoxide Radicals

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Received January 18, 1980

In 1946 Randall first noted that thiol compounds such as dithiouracil and thiourea were substrates for peroxidase.¹⁾ Recently horseradish peroxidase has been shown to catalyze the oxidation of some thiol compounds, dithiothreitol (DTT), cysteine and glutathione, in an oxygen-consuming reaction.²⁾ On the other hand superoxide radicals (O_2^-) can oxidize several thiol compounds³⁾ and thiol groups occurring in papain.⁴⁾

A thiol-containing nucleoside, 4-thiouridine (4SU) occurs in *t*-RNAs of *E. coli*,⁵⁻⁸⁾ *P. aeruginosa*⁶⁾ and so on. We reported that 4SU has an inhibitory effect on chloroplast development in greening plants.⁹⁻¹¹⁾ To examine the metabolic fate of 4SU in plants, we have studied the reactivity of 4SU in a peroxidase- H_2O_2 system and in O_2^- -generating system. We report here an enzymatic oxidation of 4SU to bis(4,4'-dithiouridine) (4SSU) with horseradish peroxidase and also the reactivity of 4SU and another thiol-containing base, 6-mercaptopurine (6MP) with O_2^- .

Oxidation of 4SU with horseradish peroxidase. Figure 1 shows the spectral change occurred upon addition of H_2O_2 to the reaction mixture consisting of acetate buffer, pH 5.0, peroxidase and 4SU. The absorption spectrum of 4SU characteristic with a maximum at 331 nm was replaced by the one with two maxima of lower intensity at 310 and 260 nm, which resembled that of authentic 4SSU prepared by the oxidation of 4SU in $KI-I_2$ solution.¹²⁾ On the addition of more H_2O_2 after 30 min of the reaction, no further changes in the spectrum were observed indicating a completed reaction. An isosbestic point appeared at 304 nm. During the course of the reaction, no significant oxygen consumption was observed when monitored by a Clark-type oxygen electrode.

We found that on incubation with a reducing agent such as DTT or 2-mercaptoethanol, authentic 4SSU was reduced to 4SU, with a yield of 92~96%. This reduction was also the case with the oxidation product of 4SU in the peroxidase- H_2O_2 reaction, but with a yield of 70~75%. These results suggest that 4SSU is a product but not a sole. The formation of 4SSU and uridine as a by-product was

FIG. 1. Spectral Shift of 4SU by Horseradish Peroxidase.

The reaction mixture contained 0.05 M acetate buffer, pH 5.0, 20 μ M 4SU, 8.5 μ M horseradish peroxidase (Sigma, type II) and 600 μ M H_2O_2 in a final volume of 2.0 ml. The reaction was started by the addition of H_2O_2 . The numbers in the figure indicate reaction times in minute.

demonstrated by thin layer chromatography using the following solvent system; upper phase of 1-butanol-acetic acid-water, 4:1:5; (*R_f* 4SSU, 0.30; uridine, 0.55; 4SU, 0.76) and by comparing UV spectra of the materials eluted from the plate with those of the authentic samples.

Since H_2O_2 has been reported to convert 4SU moiety in *t*-RNA to uridine,^{5,7)} we examined the non-enzymatic oxidation of 4SU by H_2O_2 , i.e. in the absence of peroxidase. A significant decrease in absorption at 331 nm of 4SU was accompanied by an increase at 260 nm. The result was interpreted as indicating that in the peroxidase- H_2O_2 system the enzymatic oxidation of 4SU is accompanied with its non-enzymatic oxidation to uridine. As a result, under the conditions used in Fig. 1, 40 nmol of 4SU was converted enzymatically to 17.4 nmol 4SSU and non-enzymatically to 7.1 nmol uridine.

Figure 2 shows the spectral changes in peroxidase which occurred during the course of 4SU oxidation at pH 7.0. Ferriperoxidase spectrum, which has the familiar maxima at 403, 495 and 640 nm, did not change in the presence of 4SU (curve 1). The addition of H_2O_2 brought about a spectrum having three maxima at 418, 530 and 555 nm and a shoulder between 610 and 620 nm (curve 2); the change implying the formation of peroxidase compound II.¹³⁾

FIG. 2. Formation of Horseradish Peroxidase Compound II upon Addition of 4SU and H_2O_2 .

The reaction mixture contained 0.05 M potassium phosphate, pH 7.0, 40 μM 4SU and 11.8 μM horseradish peroxidase (curve 1). Curves 2~4 represent absorption spectra at different times after the addition of 400 μM H_2O_2 to the reaction mixture: curve 2, 0 min; curve 3, 20 min; curve 4, 60 min.

During the course of reaction with 4SU, neither maximum at 543 nm nor shoulder near 579 nm appeared (curve 3~4) suggesting that the compound III is not involved in this reaction.

Generally, peroxidase catalysis is characterized by one-electron oxidation of donor molecules. The free radicals derived from the donor through the one-electron reduction of the peroxidase compounds I and II are so reactive to be extinguished rapidly by disproportionation and dimerization.¹⁴⁾ From the results that (1) the 4SU oxidation in peroxidase- H_2O_2 system did not accompany oxygen consumption, (2) peroxidase compound III was not involved in the reaction, and (3) sodium metabisulfite (40 μM), a radical scavenger, could inhibit the reaction completely, it follows that 4SU thiyl radical formed by the peroxidase reaction may dimerize itself to produce 4SSU. This reaction mechanism is different from the one proposed for the peroxidase-catalyzed oxidation of DTT where H_2O_2 was not necessary and the stoichiometry observed was 1:1 for oxygen consumed to DTT oxidized.

Reactivity of 4SU with O_2^- . Alternatively the reactivity of 4SU with O_2^- was tested by examining the inhibition of epinephrine oxidation in a xanthine-xanthine oxidase system.³⁾ No inhibition of epinephrine oxidation was observed by the addition of 2 mM 4SU in the reaction mixture. A higher concentration of 4SU interfered with the spectrophotometric assay. No cytochrome *c* reduction was

FIG. 3. Effect of 6MP Concentration on O_2^- -Induced Oxidation of Epinephrine.

The reaction mixture contained 0.05 M potassium phosphate, pH 7.8, 0.5 mM epinephrine, 0.1 mM xanthine, 5 μg of xanthine oxidase (Boehringer) and various concentrations of 6MP, in a final volume of 1 ml. The reaction was started by the addition of xanthine oxidase. *V* and *v* were the oxidation rates of epinephrine in the absence and presence of 6MP, respectively.

inhibited in the presence of 1 mM 4SU in another O_2^- -generating system, illuminated chloroplasts.¹⁶⁾ These findings indicate that 4SU does not react with O_2^- at physiological pH. However another thiol-containing nucleic acid base 6MP was reactive with O_2^- . 6MP inhibited the oxidation of epinephrine by O_2^- . Figure 3 indicates the concentration effect of 6MP on the inhibition. A half inhibition of epinephrine oxidation was found at 1.4 mM under the present conditions. From the rate constant for the reaction between O_2^- and epinephrine, $4 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$,³⁾ and the slope of V/v to the 6MP concentration, the second order rate constant for the reaction of 6MP with O_2^- was calculated to be $1.4 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$. The difference between 4SU and 6MP in the reactivity with O_2^- may be attributed to their structural difference: at pH 7.8 6MP essentially occurs in thiol form (lactim), while 4SU predominantly in thione form (lactam) because its pK_a for enolization is 8.3.⁸⁾ Thus, thiol-containing nucleoside existing in enolic form may be reactive with O_2^- as in the case with other thiol compounds.

The present results reveal that the oxidation of 4SU by peroxidase is not beyond the bounds of possibility in plant cells: however, the reaction of 4SU with O_2^- is unlikely to occur. The metabolism of 4SU in radish seedlings will be reported elsewhere.

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