Hydroxylamine Oxidoreductase from *Nitrosomonas:* Inactivation by Hydrogen Peroxide†

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ABSTRACT: Incubation of hydroxylamine oxidoreductase of *Nitrosomonas* with hydrogen peroxide resulted in the rapid and irreversible loss of the ability to catalyze the dehydrogenation of hydroxylamine in the presence of electron acceptors, such as phenazine methosulfate. The rate of the reaction was dependent on the concentration of enzyme and H_2O_2. Inactivation occurred most rapidly at pH values between 9 and 10. Inactivation of the enzyme by H_2O_2 did not result in alteration of the absorption spectrum of either the oxidized form of the enzyme or dithionite-reduced enzyme cytochromes with α maxima in the wavelength range 540–570 nm, indicating that those cytochromes were not directly involved in the dehydrogenase step. In contrast to the active enzyme, cytochromes with α maxima in the wavelength range 540–570 nm were not reducible by hydroxylamine in the inactivated enzyme. The dithionite-induced absorption maximum at 460 nm (cytochrome P 460), present in the active enzyme, was lost upon inactivation of the enzyme. This is the first direct indication of the involvement of cytochrome P 460 in the action of hydroxylamine oxidoreductase. Protection from inactivation was afforded by (a) substrates for the reduction of enzyme cytochrome, hydrazine, and *N*-methylhydroxylamine; (b) metal binding agents, KCN, 1,2-dihydroxybenzene-3,5-disulfonate, and hydroxyurea; (c) reductants, *o*-dianisidine, *p*-phenylenediamine, hydroquinone, pyrogallol, and dithiothreitol; (d) electron acceptors, phenazine methosulfate, and 2,6-dichlorophenolindophenol; and (e) the singlet oxygen trapping agent, 1,3-diphenylfuran. Scavengers of superoxide anion or hydroxyl radical did not protect the enzyme from inactivation.

Hydroxylamine oxidoreductase from the ammonia-oxidizing autotrophic bacterium *Nitrosomonas europaea* catalyzes the rapid oxidation of hydroxylamine to nitrite in the presence of a suitable electron acceptor, such as phenazine methosulfate (PMS[1]). An initial step of the reaction involves the removal of two electrons from hydroxylamine with the concomitant reduction of enzyme cytochrome (Hooper and Nason, 1965) and the production of nitroxyl, a small amount of which decomposes to nitrous oxide (Falcone et al., 1963; Ritchie and Nicholas, 1972). The subsequent production of nitrite occurs by the net addition of an atom of oxygen to HNO. Nitric oxide is a product of the reaction and a possible precursor to nitrite (Anderson, 1965; Ritchie and Nicholas, 1972), although it is also a possible product of nitrite reduction (Hooper, 1968; Ritchie and Nicholas, 1972). It has recently been shown that as much nitrate as nitrite is produced during the enzymatic oxidation of hydroxylamine but that, in the presence of diethyl dithiocarbamate (DTC), nitrite is produced, rather than nitrate, with the concomitant oxidation of DTC to bis(diethyl dithiocarbamoyl) disulfide (Hooper, A. B., Maxwell, P. C., and Terry, K. R., unpublished observations). Hydrazine is an inhibitor of hydroxylamine oxidation, is oxidized by the enzyme, and reduces enzyme-bound cytochromes (Hooper and Nason, 1965).

The enzyme has a high content of cytochrome. Approximately 30% of the dithionite-reducible cytochrome absorbancy in the wavelength range between 540 and 570 nm is reducible by either hydrazine or hydroxylamine (Falcone et al., 1963; Hooper and Nason, 1965; Rees, 1968; Ritchie and Nicholas, 1974). The enzyme contains cytochrome P 460, which is unique to the ammonia-oxidizing nitrifying bacteria (Rees and Nason, 1965; Erickson and Hooper, 1972; Ritchie and Nicholas, 1974). The absorption maximum at 460 nm of hydroxylamine oxidoreductase does not appear in the presence of hydroxylamine or hydrazine. The concentration of CO (5%), which results in a complete shift of the 460-nm peak of hydroxylamine oxidoreductase, does not inhibit the oxidation of hydroxylamine to nitrite (Hooper and Terry, 1973; Hooper et al., 1974). It has thus been considered unlikely that cytochrome P 460 has a direct role in the oxidation of hydroxylamine to nitrite.

In the present paper, we characterize the inactivation by hydrogen peroxide of the hydroxylamine-oxidizing capability of hydroxylamine oxidoreductase. After reaction of the enzyme with a tenfold excess of hydrogen peroxide, the cytochromes are 100% reducible by dithionite but not reducible by hydroxylamine or hydrazine. The 460-nm peak no longer appears in the presence of dithionite.

Experimental Procedures

Growth of Cells and Preparation of Enzyme. Nitrosomonas europaea (Schmidt strain) was grown and extracts were prepared from 8–30-g batches of cells by freezing and thawing as previously described (Hooper et al., 1972). To prepare partially purified hydroxylamine oxidoreductase, solid ammonium sulfate was added to the crude 20 000*g* supernatant fraction and the protein precipitating between 70 and 80% saturation was resuspended in 0.05 M phosphate solution, pH 7.5, and retained. The ammonium sulfate precipitation was repeated twice. The third ammonium sulfate precipitate (3AS) was dialyzed and stored at −20 °C until use. Enzyme activity remained constant for more than a year.

The following enzyme activities were found in the partially purified fraction used for the work reported here (expressed as the change in the indicated substrate or product, μmol min^{-1}

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[1] Abbreviations used are: PMS, phenazine methosulfate; DTC, diethyl dithiocarbamate; Tiron, 1,2-dihydroxybenzene-3,5-disulfonate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane (sulfate, pH 8.0); DCIP, 2,6-dichlorophenolindophenol.

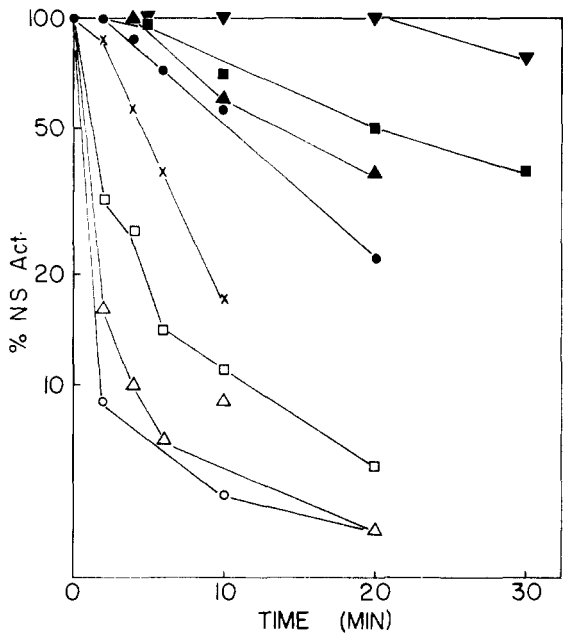

FIGURE 1: Amount of nitrite synthetase activity of hydroxylamine oxidoreductase remaining as a function of time in the presence of varying concentrations of H_2O_2. Enzyme (6×10^{-9} M) was incubated with H_2O_2 at room temperature for the indicated times and remaining activity was subsequently determined as described under Experimental Procedures. At each point, activity was compared to that of enzyme incubated in Tris without H_2O_2. H_2O_2 concentration (M) in incubation mixture: (▼) 1.25×10^{-8}; (■) 6.8×10^{-8}; (▲) 1.25×10^{-7}; (●) 1.25×10^{-6}; (X) 2.5×10^{-6}; (□) 5×10^{-6}; (△) 10^{-5}; (○) 2×10^{-5}.

mg of protein^{-1}): hydroxylamine oxidoreductase (hydroxylamine oxidized, presence of PMS), 10.3; nitrite synthetase (nitrite produced, presence of PMS), 3.5; hydroxylamine-nitrite reductase (nitrite reduced), 0.2; catalase (H_2O_2 utilized), <0.3; peroxidase (*o*-dianisidine oxidized, presence of H_2O_2), 0.31. In the case of the hydroxylamine-oxidizing and nitrite-producing enzyme, the purified fraction contained 30% of the activity present in the crude extract. Based on an approximate mol wt of 200 000 (Rees, 1968) and purity of approximately 25% estimated from sodium dodecyl sulfate gels, the concentration of hydroxylamine oxidoreductase in the stock enzyme preparation was approximately 1.25×10^{-4} M.

Chemical Assays. Nitrite was estimated by diazotization (Nicholas and Nason, 1957). Nitrate was assayed as nitrite, following reduction (Garrett and Nason, 1969). Diethyl dithiocarbamate was estimated by its reaction with the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) (Deakin et al., 1963). Hydroxylamine was estimated by reaction with 8-hydroxyquinoline (Frear and Burrell, 1964). In the concentration range 10^{-8} to 2×10^{-7} M, H_2O_2 was assayed by the fluorometric procedure of Keston and Brandt (1965).

Enzyme Assays. All incubations were at 25 °C. Routine measurement of activity of hydroxylamine oxidoreductase was carried out in a standard reaction mixture containing 100 μM hydroxylamine, 5 μM PMS, and 1.25×10^{-9} M enzyme in 0.05 M tris(hydroxymethyl)aminomethane (Tris) sulfate solution, pH 8.0, with a total volume of 5 to 15 ml. When utilized, DTC was present at a concentration of 10^{-4} M. The reaction was started by adding enzyme, and samples were withdrawn at intervals and assayed for nitrite and, as indicated, nitrate, hydroxylamine, and DTC. For peroxide inactivation, 6×10^{-9} M enzyme was incubated with H_2O_2 in Tris solution in a volume of 0.50 ml. To stop the incubation and start the measurement of enzyme activity, 2 ml of Tris solution containing 125 μM hydroxylamine and 6 μM PMS were added to the incubation mixture. H_2O_2 (30%, Mallinckrodt) was diluted in H_2O and used within 3 h. The actual H_2O_2 concentration was determined by measuring absorbancy at 240 nm in a 50 mM stock solution. Hydroxylamine-cytochrome *c* reductase and nitrite reductase were assayed as described previously (Erickson and Hooper, 1972). Hydroxylamine-DCIP reductase was measured as the rate of decrease in absorbance at 600 nm of 100 μM DCIP in the presence of the enzyme, 100 μM NH_2OH, and 5 μM PMS in 0.05 M Tris, pH 8.0. Catalase was estimated as the enzyme-dependent decrease of absorbance at 240 nm of 60 mM hydrogen peroxide (Worthington Bulletin 1.11.1.7, 1967, Worthington Biochemical Corp., Freehold, N.J.). Peroxidase was followed by measuring the increase of absorbance at 460 nm in the presence of 0.1% *o*-dianisidine, 100 μM hydrogen peroxide, and the enzyme (Worthington Bulletin 1.11.1.6, 1966, Worthington Biochemical Corp., Freehold, N.J.).

Results

Kinetics of Inactivation. As shown in Figure 1, incubation of 6×10^{-9} M hydroxylamine oxidoreductase with H_2O_2 resulted in the rapid loss of the ability of the enzyme to subsequently catalyze the aerobic oxidation of NH_2OH to HNO_2. Inactivation did not occur in the absence of H_2O_2. The kinetics of inactivation were complex. A lag period, during which enzyme activity remained constant, was observed at concentrations of hydrogen peroxide below 2.5×10^{-6} M, and was especially pronounced at a concentration of hydrogen peroxide of 1.25×10^{-8} M. Commonly, the rate of inactivation of the final 10–20% of enzyme activity was slower than the rate of inactivation of the first 70–90% of enzyme activity. Under certain conditions, inactivation was approximately first order with respect to time of incubation with H_2O_2. The $t_{1/2}$ (time for 50% inactivation) decreased with increasing concentration of H_2O_2. It is noteworthy that the lowest concentration of H_2O_2 employed was approximately twice the concentration of the enzyme. This fact and the initial lag of inactivation at very low concentrations of H_2O_2 suggested that an inactivating compound may have been generated during the incubation of the enzyme. In the presence of 1.25×10^{-7} M H_2O_2, the $t_{1/2}$ for inactivation was the same over a range of enzyme concentrations from 2.5×10^{-10} to 2.5×10^{-8} M; in the presence of 1.25×10^{-6} M H_2O_2, the $t_{1/2}$ for inactivation was the same for 6×10^{-9} and 1.25×10^{-7} M enzyme.

Incubation with H_2O_2 caused irreversible inactivation rather than simply inhibition of the enzyme, as indicated by the fact that enzyme activity was not restored following removal of H_2O_2 by either dialysis or dilution. Inclusion of 10^{-5} M H_2O_2 in the standard enzyme reaction mixture caused no change in the rate of nitrite synthesis.

Effect of Incubation Conditions. At pH values of 9 or 10, the values of $t_{1/2}$ for inactivation of the enzyme by 1 μM H_2O_2 were approximately one-fourth the corresponding values at pH values of 6, 8, or 11. At a particular pH value, the rate of inactivation varied slightly with the composition of buffer (glycine > Tris > phosphate). Inactivation did not require Cl^- ion, suggesting that the process did not involve a species of chlorine, such as free HOCl or Cl_2 as produced by myeloperoxidase (Harrison and Schultz, 1976), a complex of chloride with the enzyme as observed with chloroperoxidase (Hollenberg et al., 1974), or the formation of a chloramine as seen in the action of myeloperoxidase (Stelmaszynska and Zgliczynski, 1974). The rate of inactivation was not decreased by carrying out the reaction in complete darkness.

TABLE I: Inactivation of Hydroxylamine Oxidoreductase.[a]

Enzyme Determined	Inactivation (%)
Hydroxylamine Oxidoreductase	
Hydroxylamine oxidation	68
Nitrite synthesis	73
Nitrate synthesis	63
Hydroxylamine Oxidoreductase + DTC	
Hydroxylamine utilization	73
Nitrite synthesis	78
DTC oxidation	62
Hydroxylamine Oxidoreductase	
Mammalian cytochrome *c* reduction (presence of PMS)	63
DCIP reduction (presence of PMS)	85
Hydrazine-mammalian cytochrome *c* reductase	70
o-Dianisidine peroxidase	14

[a] Enzyme was incubated with 10^{-6} M H_2O_2, as described under Experimental Procedures. After 20 min, enzyme was stored on ice and the remaining enzyme activities were assayed as described under Experimental Procedures and compared to the equivalent activities in an aliquot of enzyme which had been incubated in the absence of H_2O_2.

Enzyme Activity Other than Nitrite Synthesis. As shown in Table I, the ability of hydroxylamine oxidoreductase to catalyze the disappearance of hydroxylamine and the production of nitrate was inactivated to the same extent as the ability to produce nitrite. Similarly, inactivation by treatment with H_2O_2 resulted in the loss of ability to catalyze the concomitant oxidation of hydroxylamine and diethyl dithiocarbamate and production of nitrite. The rates of enzymatic reduction of either mammalian cytochrome *c* or 2,6-dichlorophenolindophenol (DCIP) in the presence of a catalytic quantity of PMS and substrate quantities of either hydroxylamine or hydrazine, four separate assays for what is thought to be the same dehydrogenase step, were similarly inactivated. It therefore appeared that the enzyme was inactivated at least at the site of initial dehydrogenation of hydroxylamine. In contrast, the activity of *o*-dianisidine peroxidase, a contaminant in the enzyme fraction, was decreased by only 14%.

Changes in the Absorption Spectrum of Enzyme. As shown in Figure 2, curve c, the oxidized absorption spectra of the H_2O_2-inactivated enzyme and the fully active enzyme were identical. Curves a and d of Figure 2 are the spectra of the active enzyme in a steady state of reduction in the presence of an excess of dithionite or 10^{-4} M NH_2OH, respectively. As shown previously (Hooper and Nason, 1965; Rees, 1968; Ritchie and Nicholas, 1974), approximately 30% of the dithionite-reducible cytochromes with absorption maxima in the range 540–570 nm were reduced by NH_2OH in the untreated enzyme and the absorption maximum at 463 nm (indicative of cytochrome P 460) appeared in the presence of dithionite but not hydroxylamine. Comparison of curve e, the hydroxylamine-reduced spectrum of the inactivated enzyme, with curve d shows that inactivation with H_2O_2 resulted in the loss of reducibility of essentially all cytochromes with absorption maxima in the 540–560-nm range. The dithionite-reduced absorption spectra of the untreated (curve a) and H_2O_2-inactivated (curve b) enzyme were essentially identical in the Soret region and in the wavelength range 490–600 nm. Therefore, the change of hydroxylamine-reducibility of c-type cytochromes in the H_2O_2-inactivated enzyme was not due to

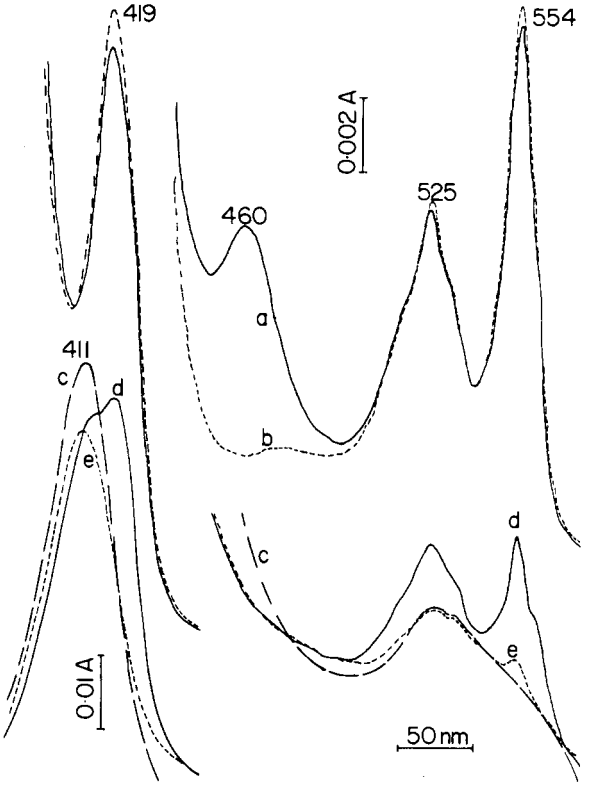

FIGURE 2: Effect of incubation with H_2O_2 on the spectral properties of hydroxylamine oxidoreductase. A sample of hydroxylamine oxidoreductase (1.25×10^{-7} M) was completely inactivated by incubation for 30 min with 5 μM H_2O_2. Absorption spectra were determined against a blank of Tris solution in an Aminco DW-2 spectrophotometer with a band-pass of 2 nm and light path of 1 cm. The sample was reduced by the addition of a few crystals of sodium dithionite or 10^{-4} M NH_2OH. (a) Control, dithionite reduced; (b) inactivated enzyme, dithionite-reduced; (c) control enzyme or inactivated enzyme, oxidized; (d) control enzyme, presence of NH_2OH; inactivated enzyme, presence of NH_2OH.

H_2O_2-dependent destruction of those cytochromes. Comparison of the 460-nm region of curves a and b shows that the 460-nm peak was not present in the dithionite-reduced spectrum of the H_2O_2-inactivated enzyme.

Protection from Inactivation. When present in the inactivation mixture, several compounds prevented inactivation of the enzyme (Table II). In most cases, the compounds had no effect on the enzyme when incubated with the enzyme in the absence of H_2O_2. In all cases, the possible disappearance of H_2O_2 under the conditions of incubation of compound, enzyme, and H_2O_2 was determined. Only with *o*-dianisidine and *p*-phenylenediamine was H_2O_2 utilized. Hydroxylamine, hydrazine, or N-methylhydroxylamine protected the enzyme completely and are each known to reduce enzyme-associated cytochromes (Falcone et al., 1963; Hooper and Nason, 1965; Rees, 1968; Ritchie and Nicholas, 1974). The presence of the metal-binding agents hydroxyurea, KCN, or Tiron also resulted in protection of the enzyme. In contrast, the presence of 1 mM EDTA, 100 μM ethyl xanthate, 100 μM *o*-phenanthroline, or 50% CO did not protect against inactivation of the enzyme. Tiron reacts with superoxide (Miller and Macdowall, 1975), suggesting the possible involvement of superoxide in the inactivation. However, the presence of superoxide dismutase (2000 units/ml) or mammalian cytochrome *c* (100 μM), which react with superoxide anion (McCord and Fridovich, 1968), did not protect the enzyme fron inactivation. As expected, the presence of 2000 units/ml of catalase completely protected the enzyme from inactivation. The presence of 1%

TABLE II: Effect of Compounds on the Inactivation of Hydroxylamine Oxidoreductase by H_2O_2.[a]

Compd	Concn (M)	Effect of Compound Alone (%)	Protection from Inactivation (%)
NH_2OH	10^{-6}		100
	10^{-7}		40
NH_2NH_2	10^{-4}	50	100
N-Methylhydroxylamine	10^{-4}	71	90
Hydroxyurea	10^{-4}	100	100
KCN	10^{-4}	100	100
1,2-Dihydroxybenzene-3,5-disulfonate (Tiron)	10^{-2}	100	100
Catalase	2000 units/ml	107	107
1,3-Diphenylfuran	10^{-4}	77	56
o-Dianisidine	4×10^{-3}	100	100
p-Phenylenediamine	10^{-4}	100	100
Hydroquinone	10^{-4}	100	100
Pyrogallol	10^{-4}	100	100
Dichlorophenolindophenol	10^{-4}	86	100
Phenazine methosulfate	10^{-4}	100	95
Dithiothreitol	10^{-4}	66	85

[a] After incubation of 6×10^{-9} M hydroxylamine oxidoreductase for 20 min at room temperature in the presence of 1 μM H_2O_2 and in the presence or absence of the indicated compounds, remaining enzyme activity was determined as described under Experimental Procedures. In each case, the remaining nitrite synthetase activity was compared to that of a control sample incubated in Tris alone. A comparison of the percent inactivation in the presence and absence of protective compound was expressed as "Protection from Inactivation". In addition, the effect on subsequent enzyme activity of incubation with the compound in the absence of H_2O_2 was determined ("Effect of Compound Alone", percent control).

bovine serum albumin was not protective. Compounds such as mannitol (10 mM), *tert*-butyl alcohol (15 mM), formate (10 mM), and ethanol (50 mM), which are known to trap hydroxyl radicals (Dorfman and Adams, 1973), did not protect against inactivation, indicating that free OH· was not involved in the inactivating reaction. Protection was not afforded the enzyme by the presence of 100 μM xanthine, which has been shown to protect against the H_2O_2-dependent inactivation of superoxide dismutase (Hodgson and Fridovich, 1975). The singlet oxygen-scavenging compound 1,3-diphenylfuran (Mayeda and Bard, 1973) gave partial protection against inactivation by H_2O_2. Four compounds which are known to be substrates for oxidase or peroxidase activity present in this enzyme preparation, 4×10^{-3} M *o*-dianisidine, 10^{-4} M *p*-phenylenediamine, 10^{-4} M hydroquinone, or 10^{-4} M pyrogallol, protected the enzyme from inactivation. The presence of 10^{-4} M PMS, DCIP, or dithiothreitol also protected the enzyme from inactivation.

Other Means of Inactivation Attributable to H_2O_2. Hydroxylamine oxidoreductase was inactivated (a) 20% following dialysis for 12 h; (b) 85% following dialysis for 16 h against a suspension of Chelex (Bio-Rad Laboratories, Richmond, Calif.); and (c) 68% following incubation for 20 min with 10^{-4} M glutathione. The presence of 1000 units/ml of catalase prevented inactivation of the enzyme by the three procedures, suggesting that inactivation had been caused by the presence of H_2O_2. Hydrogen peroxide, produced during the enzyme-catalyzed aerobic oxidation of hydroxylamine in the presence of PMS, caused inactivation of hydroxylamine oxidoreductase late in the reaction after approximately 70% of the hydroxylamine had been utilized. If hydroxylamine was continually replaced, the enzyme remained active for hours.

Discussion

Approximately 6×10^{-9} M hydroxylamine oxidoreductase from *Nitrosomonas* is shown here to undergo rapid inactivation in the presence of a tenfold excess of H_2O_2 with significant inactivation occurring with a twofold excess of H_2O_2. Inactivation of 1 μM superoxide dismutase from bovine erythrocyte has been shown by Bray et al. (1974) to occur in the presence of 1 mM H_2O_2 and to involve initial reduction of enzyme-bound copper, followed by the destruction of a histidine residue. Hodgson and Fridovich (1975) propose that hydroxyl radical, generated by reaction between H_2O_2 and enzyme-Cu^+, reacts with enzyme histidine. Cytochrome *c* peroxidase reacts with a tenfold excess of H_2O_2 in the absence of reducing substrate to undergo oxidation of 30% of the tyrosyl residues and concomitant loss of activity (Coulson and Yonetani, 1972). The reaction of a 100-fold excess of H_2O_2 with horseradish peroxidase results in the degradation of heme (Yamazaki, 1974). Vega et al. (1975) report the inactivation of nitrite reductase from *N. crassa* by 1 mM H_2O_2, and Mochan and Degn (1972) describe the destruction of ferricytochrome *c* by H_2O_2. Significantly, many of these enzymes have in common a metabolic binding site for H_2O_2. A conclusion of the present work is that hydroxylamine oxidoreductase also has a high-affinity binding site for H_2O_2.

Inactivation of hydroxylamine oxidoreductase by H_2O_2 probably involves the oxidation of part of the enzyme. The protective effect of diphenylfuran suggests the possible involvement of singlet oxygen, whereas the lack of protection by agents which will trap hydroxyl radical or superoxide anion indicates that, if such species of oxygen are involved, they must be inaccessible to the scavenging agents. A simple explanation for the protective effect of hydroxylamine, hydrazine, and *N*-methylhydroxylamine and possibly *o*-dianisidine, *p*-phenylenediamine, hydroquinone, pyrogallol, and dithiothreitol is that those compounds maintained the enzyme in the reduced form. The protective effects of KCN, hydroxyurea, and Tiron, as well as hydroxylamine, N-methylhydroxylamine, and hydrazine might occur as the compounds bind a metal at the active site of the enzyme and prevent the binding of H_2O_2. An explanation for the protective effect of PMS or DCIP, electron acceptors for the dehydrogenase portion of the reaction, is less obvious. Those compounds might trap reactive forms of oxygen or bind protectively to the enzyme.

Comparison of the dithionite-reduced absorption spectra of the active and H_2O_2-inactivated enzyme showed that cytochromes with absorption maxima in the range 540–570 nm were essentially unchanged, indicating that those cytochromes are not involved in the dehydrogenase portion of the reaction. Conversely, the dithionite-reduced absorption maximum at 460 nm (indicative of cytochrome P 460) was no longer present in the inactivated enzyme. Cytochrome P 460 is unique to the ammonia-oxidizing nitrifiers, purifies along with hydroxylamine oxidoreductase, binds CO with high affinity, and contains an unidentified heme which is not protoheme IX and is not extracted with acidic acetone from the protein (Erickson and Hooper, 1972). In the Soret region, the dithionite-reduced *plus*-CO absorption spectrum resembles that of cytochrome P 450 of mammalian microsomes. The absorption maxima of the oxidized (435 nm) and dithionite-reduced *plus* CO (450

and 460 nm) spectra of cytochrome P 460 resemble the corresponding spectra of ferrous myeloperoxidase (Odajima and Yamazaki, 1970). Prolonged incubation of a dithionite-reduced purified sample of cytochrome P 460 not associated with hydroxylamine oxidoreductase has been shown to result in the loss of the absorbancy maxima at 465 and 435 nm, resulting in a low, broad absorbancy in the 395–450-nm region (Erickson and Hooper, 1972). Because H_2O_2 is generated chemically from dithionite in aerated solutions, the dithionite-induced change in free cytochrome P 460 may be analogous to the H_2O_2-induced change in enzyme-associated cytochrome P 460 reported here.

Because the inactivated enzyme was unable to oxidize hydroxylamine in the presence of any of the electron acceptors tested or to catalyze reduction by hydroxylamine of enzyme-bound cytochrome, it appears that inactivation occurred either at the site of initial dehydrogenation of hydroxylamine or a portion of the enzyme involved in transfer of electrons from the initial site of reduction of the enzyme to another site on the enzyme. The concomitant change in spectral properties of cytochrome P 460 and loss of hydroxylamine dehydrogenase enzyme activity reported here is the first direct evidence for the involvement of cytochrome P 460 in the enzyme reaction catalyzed by hydroxylamine oxidoreductase, although clearly, the parallel losses might be coincidental.

Previous work has shown that the aerobic oxidation of hydroxylamine to nitrite in the presence of hydroxylamine oxidoreductase and PMS or in intact cells was not inhibited by CO at a concentration which caused a complete shift in the 460-nm absorption maxima of the dithionite-reduced enzyme. Taken together, these observations suggest that cytochrome P 460 is involved in the action of hydroxylamine oxidoreductase but that CO can easily be displaced from cytochrome P 460 by a molecular participant in the reaction or that cytochrome P 460 is not present in a reduced form and accessible to CO binding during the action of the enzyme.

Because measurement of the reaction in which oxygen is incorporated into HNO is dependent upon the generation of HNO by the dehydrogenase step, it is not possible to say whether the oxygen-addition step is also inactivated by H_2O_2. As a pigment which binds CO and possibly H_2O_2, an involvement of cytochrome P 460 in the oxygen-metabolizing portion of the reaction might reasonably be predicted. If so, the simultaneous alteration by H_2O_2 of the dehydrogenase and oxygen-incorporating (cytochrome P 460) sites suggests the possibility that the two sites are identical or in close physical proximity.

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