Oxidation of 2-Nitropropane by Horseradish Peroxidase

INVOLVEMENT OF HYDROGEN PEROXIDE AND OF SUPEROXIDE IN THE REACTION MECHANISM

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Incubation of aqueous solutions of 2-nitropropane in air causes a slow oxidation reaction that generates H_2O_2 . Purified horseradish peroxidase catalyses the oxidation of such preincubated 2-nitropropane solutions according to the equation:

The pH optimum is 4.5 and K_m for 2-nitropropane is 16 mM. Other nitroalkanes or nitro-aromatics tested are not oxidized at significant rates by peroxidase. H_2O_2 or 2,4-dichlorophenol increases the rate of 2-nitropropane oxidation by peroxidase. Catalase inhibits the reaction completely. Superoxide dismutase or mannitol, a scavenger of the hydroxyl radical, $\text{OH}^\cdot$, each inhibits partially. Aniline and guaiacol are also powerful inhibitors of 2-nitropropane oxidation. It is suggested that peroxidase uses the traces of H_2O_2 generated during preincubation of 2-nitropropane to catalyse oxidation of this substrate into a radical species that can reduce O_2 to the superoxide ion, $\text{O}_2^{\cdot-}$. $\text{O}_2^{\cdot-}$, or $\text{OH}^\cdot$ derived from it, then appears to react with more nitropropane, generating further radicals and H_2O_2 to continue the oxidation. Inhibition by aniline and guaiacol seems to be due to a competition for H_2O_2 .

Little (1957) reported that extracts of pea seedlings catalyse the oxidation of 2-nitropropane to acetone. The enzyme involved was not characterized, but he suggested that it might be a peroxidase. Since then, little work has been carried out on enzymes metabolizing nitroalkanes in plant tissues, even though these compounds are widely used in industry and are therefore likely environmental contaminants (Soda *et al.*, 1978). Kido *et al.* (1976) discovered a 2-nitropropane dioxygenase enzyme in extracts of a yeast that had been grown on 2-nitropropane as sole carbon source, but this enzyme has not been reported in higher plants. Certain flavoproteins, such as glucose oxidase, are also capable of oxidizing nitroalkanes, but this is certainly not their physiological function (Porter & Bright, 1977).

In the present paper, we show that purified horseradish peroxidase (donor- H_2O_2 oxidoreductase, EC 1.11.1.7) is capable of oxidizing 2-nitropropane to acetone. Since the superoxide radical, $\text{O}_2^{\cdot-}$, is involved in the oxidation of several compounds by peroxidase (Yamazaki & Piette, 1963; Yamazaki & Yokota, 1973; Halliwell, 1977; Halliwell & De Rycker, 1978) we have investigated the role of this radical species in nitropropane oxidation. The principal tool used was the enzyme superoxide dismutase (superoxide-superoxide oxidoreductase, EC 1.15.1.1),

which catalyses a rapid dismutation of $\text{O}_2^{\cdot-}$ into H_2O_2 and O_2 as shown in eqn. (1):

and is thus a powerful inhibitor of $\text{O}_2^{\cdot-}$ -dependent reactions (Fridovich, 1975; Halliwell, 1978a).

Materials and Methods

Reagents

Horseradish peroxidase (type VI) was obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. It was dissolved in 10 mM- KH_2PO_4 buffer adjusted to pH 4.5 with KOH and its concentration calculated from its A_{403} (Sawada & Yamazaki, 1973). The ratio $A_{403}:A_{278}$ was 2.90, which means that the enzyme is almost completely pure (Sawada & Yamazaki, 1973). 2-Nitropropane and 2-nitropropanol were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Catalase (H_2O_2 - H_2O_2 oxidoreductase, EC 1.11.1.6) was obtained from Boehringer Corp., London W.5, U.K., and found to be free of superoxide dismutase activity (Halliwell, 1973). Superoxide dismutase prepared by the method of McCord & Fridovich (1969) was purchased from Sigma. It did not contain catalase activity. Where in-

licated, it was denatured by heating at 100°C for 10 min and cooling before use. All other reagents were purchased from Sigma or from BDH Chemicals, London, U.K.

Methods

Determinations. NO_2^- was determined by a specific colorimetric method (Ida & Morita, 1973). Acetone and 2-nitropropane were measured by g.l.c. on a 6.85 g Porapak type Q (100–120 mesh) column at 130°C. The retention time for acetone was 13.8 min and that for 2-nitropropane was 78.6 min.

Enzyme assays. Superoxide dismutase was assayed by the cytochrome *c* method: 1 unit inhibits the reduction of cytochrome *c* by 50% under the assay conditions of McCord & Fridovich (1969). Catalase was assayed by the fall in A_{240} (Luck, 1963): 1 unit is the amount that catalyses removal of 1 μmol of H_2O_2 /min under these conditions. 2-Nitropropane oxidation by peroxidase was usually assayed at 20°C by following O_2 uptake with a Hansatech oxygen electrode (Hansatech, King's Lynn, Norfolk, U.K.) calibrated according to the manufacturer's instructions. Reaction mixtures contained, in a total volume of 2 ml, the following reagents at the final concentrations stated: 2-nitropropane (40 mM), KH_2PO_4 (0.2 M adjusted to pH 4.5 with KOH) and peroxidase (9 nM). A nitropropane solution (0.2 M) was made up by adding 0.89 g of 2-nitropropane to 50 ml of 0.2 M- KH_2PO_4 , pH 6.8, and mixing in a sealed vessel on a magnetic stirrer at room temperature for 3 h. The solution was then adjusted to pH 4.5 by adding HCl and used to make up the above reaction mixture. Preincubation of the substrate in this way was found to be necessary to obtain maximum rates of 2-nitropropane oxidation, as observed by Little (1957).

Results

Oxidation of 2-nitropropane by peroxidase

Rapid oxidation of solutions of 2-nitropropane preincubated as described in the Materials and Methods section could easily be demonstrated in the presence of peroxidase by measuring O_2 uptake with an O_2 electrode. The rate of O_2 uptake was too slow to measure in the absence of peroxidase or if peroxidase was replaced by equimolar concentrations of haemoglobin, myoglobin, haematin, catalase or cytochrome *c*. The rate of 2-nitropropane oxidation was proportional to the concentration of peroxidase present in the range used in these experiments.

Fig. 1 shows the effect of pH on 2-nitropropane oxidation by peroxidase. The pH optimum was 4.5. At this pH, the reaction products were examined. Table 1 gives some typical results that show that, per mol of 2-nitropropane oxidized, 1 mol of acetone

Fig. 1. Effect of pH on the rate of 2-nitropropane oxidation by peroxidase

Reaction mixtures were as described in the Materials and Methods section, except that the 0.2 M- KH_2PO_4 buffer was adjusted to the pH indicated with KOH. The effect of pH was also examined by assaying the reaction mixtures for NO_2^- . Symbols: ■, uptake of O_2 ($\mu\text{mol}/\text{min}$); ●, NO_2^- formation ($\mu\text{mol}/30\text{s}$).

and 1 mol of NO_2^- were formed, and 0.5 mol of O_2 was taken up. Hence the equation for the reaction may be written as:

The oxidation reaction obeyed the Michaelis-Menten equation. The K_m for 2-nitropropane was found to be 16 ± 2 mM (mean $\pm$ s.d. of five determinations) by using direct linear plots (Eisenthal & Cornish-Bowden, 1974) and also Lineweaver-Burk plots (Fig. 2 shows a typical result).

Table 2 shows that the oxidation reaction was highly specific for 2-nitropropane. Of the other compounds tested, only nitroethane and 2-nitropropanol gave significant rates of O_2 uptake or NO_2^- formation. As might be expected, the peroxidase inhibitors azide and KCN completely inhibited oxidation (Table 3). Nitroethane, 4-nitrophenol, *p*-cresol, aniline and guaiacol also inhibited 2-nitropropane oxidation (Table 3). The inhibitions by aniline and guaiacol were found to be competitive (Fig. 2).

Catalase inhibited O_2 uptake by the peroxidase-2-nitropropane system whether it was added at the beginning of the reaction or during it (Fig. 3). An equal amount of bovine serum albumin did not inhibit. Hence H_2O_2 is required for nitropropane oxidation, but it does not accumulate in the reaction mixture, since there is no 'burst' of O_2 release when catalase is added during the course of the reaction (Fig. 3). Catalase also completely inhibited nitropropane oxidation as assayed by acetone or NO_2^-

Table 1. *Stoichiometry of 2-nitropropane oxidation by peroxidase*

Assays and determinations were carried out at pH4.5, in a reaction volume of 2ml, as described in the Materials and Methods section. Incubations were carried out for periods varying from 30 to 240min and corrections have been made for any non-enzymic rates. —, Not determined.

Expt. no.	2-Nitropropane used (μmol)	Amounts of:		
		O ₂ taken up (μmol)	NO ₂ ⁻ produced (μmol)	Acetone produced (μmol)
1	1.60	0.77	1.60	1.58
2	1.75	0.84	—	1.75
3	—	0.30	—	0.62
4	0.72	0.37	—	0.71
5	1.41	0.67	1.40	—
6	—	0.51	0.96	—
7	—	0.36	0.82	—
8	0.51	0.26	0.54	0.52
9	1.29	—	1.32	1.23
10	1.75	—	1.87	1.71

Fig. 2. *Effects of guaiacol and aniline on the kinetics of 2-nitropropane oxidation by peroxidase*

The initial rate of 2-nitropropane oxidation was determined at various substrate concentrations as described in the Materials and Methods section and the results are presented as a Lineweaver-Burk plot. Symbols: ●, no inhibitor added; ■, 0.02 mM-aniline present; ▲, 0.17 mM-guaiacol present.

formation. In contrast, addition of H₂O₂ increased the rate of oxidation (Table 3).

To obtain maximum rates of 2-nitropropane oxidation by peroxidase, the substrate has to be incubated with buffer for 3 h as described in the Materials and Methods section. Such preincubation is necessary even if the substrate has been thoroughly dispersed in the buffer by sonication. However, addition of H₂O₂ (0.42 mM) was found to allow significant oxidation of even a freshly prepared solution of 2-nitropropane by peroxidase. This suggests that traces of H₂O₂ generated by autooxidation of 2-nitropropane during the preincubation are necessary for the subsequent peroxidase-catalysed oxidation. In agreement with this, preincubation of 2-nitropropane under an

atmosphere of N₂ for 3 h decreased the rate of oxidation on subsequent addition of peroxidase to 0.2 % of that seen when the substrate had been preincubated in air. Also, if preincubation was carried out in the presence of a sealed, leakproof dialysis sac containing 10 mg of Boehringer catalase, the rate of oxidation on subsequent addition of peroxidase (after removal of the catalase) was only 31 % of normal. All other experiments described in the Results section were carried out with 2-nitropropane preincubated in air for 3 h. The autooxidation of 2-nitropropane during preincubation was not light-sensitive: the same rates of O₂ uptake were observed on subsequent addition of peroxidase whether the flasks had been exposed to normal room light or kept in the dark.

Addition of superoxide dismutase (60 units) to the peroxidase-2-nitropropane system caused a 30–35 % inhibition of O₂ uptake, NO₂⁻ formation or acetone formation, whether it was present at the beginning of the reaction or added during it (Fig. 3). Increased amounts of dismutase did not produce any greater inhibition and heat-denatured enzyme had no effect.

O₂⁻ and H₂O₂ react together, by an unknown mechanism, to produce the hydroxyl radical, OH[•] (Fridovich, 1975; Halliwell, 1978a). The effect of the OH[•]-radical scavenger mannitol on 2-nitropropane oxidation was therefore examined: 100 mM-mannitol inhibited oxidation by 25–30 % (Table 3), whereas 100 mM-KCl had no effect. Inhibitions by superoxide dismutase and by mannitol were not additive: addition of 100 mM-mannitol together with 60 units of dismutase produced no greater inhibition than was observed in the presence of either inhibitor alone. It was not possible to test other commonly used scavengers of OH[•] radicals (e.g. ethanol, formate, thiourea), as they may well be substrates for peroxidase in this system.

Table 2. *Specificity of peroxidase for 2-nitropropane oxidation*

Oxidation was measured by O₂ uptake as described in the Materials and Methods section. The other substrates listed below were preincubated by the procedure used for nitropropane and equal amounts were tested in the assay. Similar results were obtained when oxidation was tested for by measuring NO₂⁻ formation.

Compound tested	O ₂ taken up (μ mol/min)	Rate of O ₂ uptake (% of that with 2-nitropropane)
2-Nitropropane	1.44	100
2-Nitropropanol	0.11	7.6
Nitroethane	0.02	1.4
Nitromethane	0	0
Tetranitromethane	0	0
Nitrobenzene	0	0
Aniline	0	0
Guaiacol	0	0
<i>p</i> -Nitroaniline	0	0
2-Nitrobenzoic acid	0	0
4-Nitrophenol	0	0
Nitropropane	0.004	0.3

Table 3. *Effect of reagents on 2-nitropropane oxidation by peroxidase*

Oxidation was measured by O₂ uptake as described in the Materials and Methods section. Similar results were obtained when it was measured by NO₂⁻ formation.

Compound added	Concn. (mM)	Rate of oxidation	
		(μ mol of O ₂ taken up/min)	(% of rate in absence of addition)
None	—	1.94	100
NaN ₃	2	0	0
KCN	2	0	0
Nitroethane	10	1.40	72
	20	0.86	44
Nitrophenol	10	1.45	75
	25	0.97	50
Guaiacol	2	0.19	10
Mannitol	10	1.75	90
	100	1.45	75
KCl	100	1.94	100
2,4-Dichlorophenol	0.5	5.04	260
<i>p</i> -Coumaric acid	0.5	1.59	82
<i>p</i> -Cresol	0.5	0.49	25
H ₂ O ₂	0.42	2.72	140
H ₂ O ₂ + 2,4-dichlorophenol	0.42 + 0.5	14.44	744
H ₂ O ₂ + <i>p</i> -coumaric acid	0.42 + 0.5	9.32	480
H ₂ O ₂ + <i>p</i> -cresol	0.42 + 0.5	3.08	159

Effect of peroxidase cofactors on 2-nitropropane oxidation

Mn²⁺ and certain phenols often act as cofactors for peroxidase-catalysed reactions (e.g. Halliwell & De Rycker, 1978), and their effects on 2-nitropropane oxidation were therefore examined. MnCl₂ (1 mM) increased the rate of oxidation, but this was found to be almost completely due to a non-enzymic breakdown of 2-nitropropane induced by Mn²⁺. This breakdown could be completely inhibited by superoxide dismutase (50 units). Mn²⁺ has previously been shown to promote the non-enzymic breakdown

of dihydroxyfumarate (Halliwell, 1977) and of NADH (Halliwell, 1978*b*) by interacting with O₂^{-•}.

Of the phenols tested, only 2,4-dichlorophenol increased the rate of nitropropane oxidation by peroxidase (Table 3). In the presence of added H₂O₂, however, *p*-coumaric acid (4-hydroxycinnamic acid) and *p*-cresol gave further stimulations and the effect of 2,4-dichlorophenol was markedly increased.

Spectral studies on the interaction of 2-nitropropane with peroxidase

Addition of preincubated solutions of 2-nitropropane (to a final concentration of 20 mM) to peroxi-

Fig. 3. Effects of catalase and superoxide dismutase on 2-nitropropane oxidation by peroxidase

The results presented are oxygen-electrode traces, but identical results were obtained when nitropropane oxidation was measured by NO₂⁻ or acetone formation. Reaction mixtures were as described in the Materials and Methods section. Curve (a), complete; curve (b), plus 60 units of superoxide dismutase; curve (c), as curve (a), but 60 units of dismutase added at the arrow; curve (d), plus 5000 units of catalase; curve (e), as curve (a), but catalase (5000 units) injected at the arrow. Addition of greater quantities of superoxide dismutase had no further inhibitory effect.

dase solutions (2.3 μM) at pH 4.5 did not cause any change in the absorption spectrum of the enzyme. However, when H₂O₂ (0.42 mM) was added to peroxidase solutions, the spectrum of peroxidase Compound I appeared, in agreement with previous results (Keilin & Hartree, 1951). Subsequent addition of 2-nitropropane caused a gradual loss of Compound I and formation of free peroxidase, with the intermediate formation of peroxidase Compound II.

Discussion

In the present paper we have shown that purified preparations of the plant enzyme horseradish peroxidase can oxidize 2-nitropropane to acetone and nitrite. H₂O₂ is also formed in the reaction mixture and the effect of catalase shows it to be essential for 2-nitropropane oxidation, but it does not accumulate. O₂ is taken up, O₂^{•-} is formed and plays a role in the oxidation.

In agreement with the observations of Little (1957), we found that preincubation of the substrate is necessary to achieve maximum rates of 2-nitro-

propane oxidation by peroxidase. The low rate of oxidation of freshly prepared solutions was increased by adding H₂O₂, suggesting that what happens during preincubation is a very slow non-enzymic breakdown of the substrate to generate traces of H₂O₂, which can be used by peroxidase to catalyse nitropropane oxidation. Removal of O₂, or the presence of catalase to destroy the H₂O₂ formed, stops this from happening. Such a mechanism has already been established for the oxidations of dihydroxyfumarate (Yamazaki & Piette, 1963; Halliwell, 1977) and NADH (Yamazaki & Yokota, 1973; Halliwell, 1978b) by peroxidase. In fact, autoxidation of hydrocarbons is a well-established reaction. For example, the autoxidation of propan-2-ol under similar conditions was developed in the U.S.A. by Shell Petroleum Co. as a means of manufacturing H₂O₂.

Peroxidase is converted by H₂O₂ into a species (Compound I) that has a nominal oxidation state of Fe(V). The enzyme usually returns to the 'resting' state, Fe(III), by catalysing two one-electron oxidations of a substrate with the intermediate formation of peroxidase Compound II. If one applies such a scheme to the oxidation of 2-nitropropane (NP), one can write a mechanism consistent with the observed spectral data as follows:

where NP[•] is a radical formed by loss of one electron from NP. It seems likely that NP[•] can transfer an electron to O₂ to make O₂^{•-} (eqn. 6), so accounting for O₂ uptake and O₂^{•-} generation in the reaction mixture. Some of this O₂^{•-} may then interact with the substrate to cause further formation of NP[•] (eqn. 7):

This would account for the observation that superoxide dismutase partially inhibits O₂ uptake, acetone formation and nitrite formation. An analogous mechanism has been proposed to account for dihydroxyfumarate oxidation by peroxidase (Halliwell, 1977). However, at pH 4.5 the non-enzymic dismutation of O₂^{•-} (eqn. 1) proceeds rapidly and would compete with reaction (7), which may well explain why there is only a small inhibition of 2-nitropropane oxidation by superoxide dismutase. It also seems that reaction (6) is much faster than the self-reaction of NP[•] radicals (eqn. 8):

since an almost stoichiometric amount of acetone is formed.

It might be $\text{OH}^\bullet$ radicals derived from $\text{O}_2^{\cdot -}$ rather than $\text{O}_2^{\cdot -}$ itself that attack the nitropropane, since there is a partial inhibition by mannitol which is not additive to that observed with superoxide dismutase. Reactions (1) and (7) both generate H_2O_2 , which would be used by the peroxidase to catalyse further oxidation of 2-nitropropane. Hence H_2O_2 is formed in the reaction mixture and is essential for the oxidation, but it does not accumulate and the catalytic quantities of H_2O_2 formed during preincubation are sufficient to trigger the reaction.

A wide range of other nitro compounds was tested (Table 2), but none was significantly oxidized by peroxidase under these conditions. However, compounds such as aniline, guaiacol and nitrophenol are well known to be oxidized by peroxidase when H_2O_2 is added (Chance & Maehly, 1955; Danner *et al.*, 1973; Dunford & Cotton, 1975; Harson & Dunford, 1976). Even when H_2O_2 (0.42 mM) was added to the reaction mixtures to ensure oxidation of these phenols no O_2 uptake or NO_2^- formation was observed (results not shown). Hence the radicals known to be produced during oxidation of these compounds cannot reduce O_2 to $\text{O}_2^{\cdot -}$ (and so cannot make additional H_2O_2) and do not break down to give NO_2^- . Inhibition of 2-nitropropane oxidation by such compounds may be due to their being preferentially oxidized by the enzyme, so using up the traces of H_2O_2 present in the 2-nitropropane and preventing its further oxidation. This would account for the competitive kinetics (Fig. 3). The stimulation of nitropropane oxidation by 2,4-dichlorophenol may be due to the ability of this phenol to react with ferropoxidase (Halliwell, 1977, 1978b).

It is interesting to compare the properties of peroxidase in nitropropane oxidation with those of the yeast nitropropane dioxygenase described by Kido *et al.* (1976) and Soda *et al.* (1978). The K_m values for nitropropane oxidation by both systems are similar, but the yeast enzyme contains FAD and non-haem iron and has a pH optimum of 8 (peroxidase, pH 4.5; see Fig. 1). Nitroethane is a good substrate for dioxygenase, but not for peroxidase. Finally, superoxide dismutase inhibits the peroxidase system by 30–35%, but the dioxygenase by more than 80%.

Peroxidases are widespread in the plant kingdom and their substrates *in vivo* are often unknown (Halli-

well, 1978a). It may be that plant peroxidase activity is involved in the metabolism of 2-nitropropane supplied to plants by environmental pollution and that a peroxidase was responsible for the results of Little (1957).

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