Hydroxylation of *p*-Coumaric Acid by Horseradish Peroxidase

THE ROLE OF SUPEROXIDE AND HYDROXYL RADICALS

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1. In the presence of dihydroxyfumarate, horseradish peroxidase catalyses the conversion of *p*-coumaric acid into caffeic acid at pH 6. This hydroxylation is completely inhibited by superoxide dismutase. 2. Dihydroxyfumarate cannot be replaced by ascorbate, H_2O_2 , NADH, cysteine or sulphite. Peroxidase can be replaced by high (10 mM) concentrations of FeSO_4 , but this reaction is almost unaffected by superoxide dismutase. 3. Hydroxylation by the peroxidase/dihydroxyfumarate system is completely inhibited by low concentrations of Mn^{2+} or Cu^{2+} . It is proposed that this is due to the ability of these metal ions to react with the superoxide radical $\text{O}_2^{\cdot-}$. 4. Hydroxylation is partially inhibited by mannitol, Tris or ethanol and completely inhibited by formate. This seems to be due to the ability of these reagents to react with the hydroxyl radical $\cdot\text{OH}$. 5. It is concluded that $\text{O}_2^{\cdot-}$ is generated during the oxidation of dihydroxyfumarate by peroxidase and reacts with H_2O_2 to produce hydroxyl radicals, which then convert *p*-coumaric acid into caffeic acid.

Horseradish peroxidase (donor- H_2O_2 oxidoreductase, EC 1.11.1.7) readily catalyses the oxidation of dihydroxyfumaric acid (Swedin & Theorell, 1940; Chance, 1952), and a mixture of peroxidase and dihydroxyfumarate can hydroxylate a wide range of aromatic compounds (Buhler & Mason, 1961; Daly & Jerina, 1970). The mechanism of this hydroxylation has not been established. However, it is known that a non-enzymic system generating the superoxide radical, $\text{O}_2^{\cdot-}$, can hydroxylate aromatic compounds (Ravindranath *et al.*, 1974). This radical species is readily formed in biological systems, and there is evidence that many enzyme-catalysed hydroxylations involve free or bound $\text{O}_2^{\cdot-}$ (Fridovich, 1974; Halliwell, 1974; Bors *et al.*, 1974; Liu *et al.*, 1974; Sligar *et al.*, 1974).

Yamazaki & Piette (1963) proposed that $\text{O}_2^{\cdot-}$ was involved in several reactions catalysed by horseradish peroxidase. Yamazaki & Yamazaki (1973) showed that the enzyme superoxide dismutase ($\text{O}_2^{\cdot-}$ - $\text{O}_2^{\cdot-}$ oxidoreductase, EC 1.15.1.1), which catalyses a rapid breakdown of $\text{O}_2^{\cdot-}$ to H_2O_2 and triplet O_2 (McCord & Fridovich, 1969), caused a transient inhibition of the oxidation of dihydroxyfumarate by horseradish peroxidase, indicating the involvement of $\text{O}_2^{\cdot-}$. Further, $\text{O}_2^{\cdot-}$ can be produced during the breakdown of peroxidase compound III (Rotilio *et al.*, 1975), which is formed during oxidation of dihydroxyfumarate (Chance, 1952).

We therefore decided to examine the role of $\text{O}_2^{\cdot-}$ in hydroxylation of aromatic compounds by the peroxidase/dihydroxyfumarate system. Since we are interested in the metabolism of *p*-coumaric acid (4-hydroxycinnamic acid), this compound was chosen

as substrate for hydroxylation, but similar results were obtained with *p*-hydroxybenzoic acid or salicylic acid.

Materials and Methods

Materials

Dihydroxyfumaric acid was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Catalase, xanthine oxidase and horseradish peroxidase were obtained from the Boehringer Corporation, London W.5, U.K. Horseradish peroxidase of the highest purity offered was also purchased from BDH Chemicals, Poole, Dorset, U.K., and from the Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. There was no difference in the ability of peroxidase from these three sources to catalyse the hydroxylation reaction. None of these preparations contained catalase activity. Superoxide dismutase activity was detected in concentrated solutions of all three preparations, but the amount present in the small quantity of peroxidase added to the assay system for hydroxylation was negligible (<0.01 unit).

Other reagents were of the highest purity available from BDH Chemicals or from Sigma Chemical Co.

Assay of hydroxylation reactions

A mixture of *p*-coumaric acid ($2.5\text{ }\mu\text{mol}$), dihydroxyfumaric acid ($30\text{ }\mu\text{mol}$) and KH_2PO_4 ($8.3\text{ }\mu\text{mol}$) was adjusted to pH 6 with KOH and incubated under air in a total volume of 1.00 ml at 25°C for 30 min in the presence of 0.02 ml of horseradish

peroxidase. This amount of enzyme solution catalysed an absorbance change of 0.036 min^{-1} at 436 nm when assayed by the guaiacol method (Chance & Maehly, 1955).

The rate of hydroxylation was constant for 30 min and then began to fall. Controls were run from which enzyme was omitted.

After 0.5 h the reaction was stopped by adding $10 \mu\text{l}$ of conc. HCl, and the products were extracted into diethyl ether. A sample of the upper layer was taken, the ether removed by evaporation on a water bath at 50°C and the residue dissolved in 1.00 ml of water. The caffeic acid present was assayed by a modification of the technique of Nair & Vaidyanathan (1964) for detecting *o*-dihydric phenols. To the 1.00 ml solution was added 0.50 ml of 0.5 M -HCl containing 40 mg of trichloroacetic acid, followed by 1.00 ml of 10% (w/v) sodium tungstate and 1.00 ml of 0.5% (w/v) NaNO_2 . After 5 min 2 ml of 0.5 M -KOH was added, and after a further 15 min the absorbance at 510 nm was measured.

A small amount of dihydroxyfumaric acid was extracted into the ether and produced a brown colour when tungstate was added during the above procedure. This colour faded rapidly when NaNO_2 was added, however, and the presence of this small amount of dihydroxyfumarate had no effect on the final colour obtained.

Standard curves were obtained by ether extraction of solutions containing caffeic acid and $10 \mu\text{l}$ of conc. HCl, together with the amounts of dihydroxyfumarate and *p*-coumaric acid present in the reaction mixtures for assay of hydroxylation. The absorbance at 510 nm was proportional to the amount of caffeic acid present in the range $0\text{--}0.2 \mu\text{mol}$.

None of the inhibitors used in the experiments reported below interfered with colour development in this assay.

Enzyme assays

Catalase ($\text{H}_2\text{O}_2\text{--H}_2\text{O}_2$ oxidoreductase, EC 1.11.1.6) was assayed by the decrease in absorbance at 240 nm as H_2O_2 was decomposed (Luck, 1963). One unit is defined as that amount catalysing the breakdown of $1 \mu\text{mol}$ of $\text{H}_2\text{O}_2/\text{min}$ under these conditions.

Superoxide dismutase (obtained from the Sigma Chemical Co.) was assayed by its ability to inhibit the reduction of cytochrome *c* by $\text{O}_2^{\cdot-}$ generated by a xanthine/xanthine oxidase system (McCord & Fridovich, 1969). One unit is that amount which causes a 50% inhibition of cytochrome *c* reduction.

Assay of dihydroxyfumarate oxidation

Oxidation of dihydroxyfumarate in the reaction mixtures used for hydroxylation was followed by the decrease in absorbance at 300 nm (Goscin &

Fridovich, 1972). Samples ($10 \mu\text{l}$) were removed from the reaction mixtures at intervals, and each was added to 10 ml of water and the absorbance recorded at once. Although *p*-coumaric acid and caffeic acid also absorb light at 300 nm , their molar extinction coefficients at this wavelength were found to be almost identical. In any case, only $2\text{--}3\%$ of the *p*-coumaric acid present was converted into caffeic acid. Hence the fall in absorbance at 300 nm could be attributed to dihydroxyfumarate oxidation.

Results

A mixture of peroxidase and dihydroxyfumarate catalysed the hydroxylation of *p*-coumaric acid at pH 6, the optimum pH for this system (Buhler & Mason, 1961). It was found that $50\text{--}70 \text{ nmol}$ of *p*-coumaric acid was hydroxylated during a 30 min incubation. Hydroxylation was abolished by omitting dihydroxyfumarate from the reaction mixtures. A low rate of hydroxylation ($3\text{--}4 \text{ nmol}$ in 30 min) was still observed if peroxidase was omitted; this has been corrected for in all the results presented below.

The product of the peroxidase-catalysed reaction co-chromatographed with authentic caffeic acid (3,4-dihydroxycinnamic acid) in the two solvent systems used by Halliwell (1975) and gave the same colour with diazotized sulphanilic acid. There was no destruction of added caffeic acid on incubation with the reaction mixtures for hydroxylation, so the results obtained are a true measure of the amount of hydroxylation achieved.

Dihydroxyfumarate could not be replaced by equimolar concentrations of ascorbate, cysteine, sulphite or NADH, or by H_2O_2 ($0.1\text{--}10 \text{ mM}$). In agreement with the results obtained by Goscin & Fridovich (1972), FeSO_4 could replace peroxidase, but 10 mM iron salt had to be present to achieve a comparable rate of hydroxylation. The chemical ascorbate/ Fe^{2+} /EDTA system described by Udenfriend *et al.* (1954) was also found to hydroxylate *p*-coumaric acid, although at lower rates ($16\text{--}20 \text{ nmol}$ of caffeic acid formed in 30 min).

Inhibition of hydroxylation

Hydroxylation of *p*-coumaric acid by the peroxidase/dihydroxyfumarate system could be completely inhibited by catalase, Mn^{2+} , Cu^{2+} or superoxide dismutase (Table 1), but neither EDTA ($0.10\text{--}10.0 \text{ mM}$) nor H_2O_2 ($0.10\text{--}2.0 \text{ mM}$) had any effect. Preliminary studies showed that the catalase used in these experiments contained no superoxide dismutase activity (see Halliwell, 1973), nor did the dismutase contain catalase activity. Thermal denaturation of catalase or dismutase greatly decreased or abolished their inhibitory effects.

Table 1. Effect of various reagents on hydroxylation of *p*-coumaric acid by peroxidase and dihydroxyfumarate

The assays of hydroxylation and dihydroxyfumarate oxidation were as described in the Materials and Methods section. The rate of dihydroxyfumarate oxidation was constant for 15 min and then began to fall slightly. The percentage rates given below are calculated from initial rates of change of absorbance at 300 nm: 100% corresponded to a change of 0.010 min^{-1} . Mn^{2+} induced a slow non-enzymic breakdown of dihydroxyfumarate; the results presented have been corrected for this. Where indicated, enzymes were denatured by heating at 100°C for 30 min. The concentrations of reagents stated below were the final concentrations in the 1.00 ml reaction mixtures.

Reagent added	Concn. of reagent added	Caffeic acid formed (nmol/30 min)	% rate of <i>p</i> -coumarate hydroxylation	% rate of dihydroxyfumarate oxidation
None	—	60	100	100
CuSO_4	0.01 mM	18	30	—
	0.02 mM	6	10	—
	0.10 mM	0	0	60
MnCl_2	0.01 mM	40	67	—
	0.05 mM	12	20	—
	0.10 mM	6	10	—
	0.20 mM	0	0	175
Superoxide dismutase	1 unit	27	45	—
	2 units	12	20	—
	5 units	3	5	—
	10 units	0	0	80
Denatured superoxide dismutase	5 units*	51	85	98
	10 units*	47	78	95
Catalase	500 units	23	38	—
	1000 units	12	20	20
	1250 units	0	0	14
Denatured catalase	1250 units*	60	100	100
Mannitol	50 mM	46	77	100
	100 mM	36	60	100
Ethanol	50 mM	36	60	100
	100 mM	32	53	100
Tris	50 mM	35	58	98
	100 mM	22	37	92
Formate	10 mM	41	68	—
	20 mM	29	48	—
	50 mM	5	8	76
	100 mM	0	0	50

* Activity before heating of the enzyme.

In contrast, superoxide dismutase (10 units), Cu^{2+} (0.1 mM) or Mn^{2+} (0.1 mM) had little or no effect on hydroxylation of *p*-coumaric acid by the FeSO_4 /dihydroxyfumarate system mentioned above (also see Goscin & Fridovich, 1972) or by the ascorbate/ Fe^{2+} /EDTA system.

Inhibition of dihydroxyfumarate oxidation

The effect of inhibitors of hydroxylation on the oxidation of dihydroxyfumaric acid by peroxidase was examined. Table 1 shows that 1000 units of catalase inhibited the oxidation severely, in agreement with the results obtained by Swedin & Theorell (1940). In contrast, amounts of superoxide dismutase that completely stopped hydroxylation had only a slight inhibitory effect on the oxidation of dihydroxyfumaric acid in these reaction mixtures. Similar results were

obtained with Cu^{2+} , whereas Mn^{2+} , a powerful inhibitor of hydroxylation, stimulated dihydroxyfumarate oxidation. It is therefore clear that, except in the case of catalase, inhibition of hydroxylation by these reagents cannot be attributed to an inhibition of dihydroxyfumarate oxidation.

Free metal ions catalyse the breakdown of $\text{O}_2^{\cdot-}$ radicals under certain conditions (Rabani *et al.*, 1973; Brigelius *et al.*, 1974; Lumsden & Hall, 1975), suggesting that the inhibition of hydroxylation at pH 6 by Cu^{2+} and Mn^{2+} might also be due to removal of $\text{O}_2^{\cdot-}$. Fig. 1 shows that Cu^{2+} and Mn^{2+} inhibited the reduction of cytochrome *c* by $\text{O}_2^{\cdot-}$ generated by a xanthine/xanthine oxidase system at pH 6. There was a close correlation between the degree of inhibition of cytochrome *c* reduction at various concentrations of metal ion and the inhibition of hydroxylation by these concentrations (compare Table 1 and Fig. 1).

Fig. 1. Effect of metal ions on reduction of cytochrome *c* by $O_2^{\cdot-}$ at pH 6

The assay conditions were those described by McCord & Fridovich (1969), except that phosphate buffer at pH 6 was used and EDTA was omitted. The reaction was initiated by adding xanthine oxidase, and the reduction of cytochrome *c* was followed at 550 nm. The concentrations of metal ions stated were the final concentrations present in the 3.00 ml reaction mixtures. Formation of uric acid was followed at 290 nm in reaction mixtures from which cytochrome *c* had been omitted. ●, No addition; ▼, plus 1 unit of superoxide dismutase; ■, plus $CuSO_4$ in the concentrations stated; ▲, plus $MnCl_2$ in the concentrations stated.

These concentrations of Cu^{2+} or Mn^{2+} did not affect urate production in this assay system, and so inhibition of cytochrome *c* reduction was not due to an inhibition of xanthine oxidase.

Inhibition of hydroxylation by scavengers of hydroxyl radicals

The effect of reagents which can react with the hydroxyl radical $\cdot OH$ on the hydroxylation of *p*-coumaric acid was examined. Table 1 shows that formate, which converts $\cdot OH$ into $O_2^{\cdot-}$ (Bors *et al.*, 1974), could completely inhibit hydroxylation at a concentration of 0.1 M. This concentration decreased the oxidation of dihydroxyfumarate in these reaction mixtures by only 50%. Mannitol (0.1 M) (McCord &

Fridovich, 1973) or ethanol (0.1 M) (Beauchamp & Fridovich, 1970) had no effect on the oxidation of dihydroxyfumarate, but inhibited hydroxylation by about 50%. Tris (0.1 M) (Paschen & Weser, 1975) inhibited dihydroxyfumarate oxidation by less than 10%, but produced a much more severe inhibition of hydroxylation. Addition of KCl (0.1 M) to the reaction mixtures did not inhibit hydroxylation, suggesting that the above effects were not due to increased ionic strength.

Discussion

During the oxidation of dihydroxyfumaric acid by horseradish peroxidase, *p*-coumaric acid can be converted into caffeic acid. The oxidation of dihydroxy-

fumarate is dependent on H_2O_2 , and so is inhibited by catalase (Swedin & Theorell, 1940; Yamazaki & Piette, 1963). As the activities of catalase required to achieve inhibition of oxidation and hydroxylation were almost identical (see Table 1), it is likely that catalase prevents hydroxylation by removing the H_2O_2 necessary for oxidation of dihydroxyfumarate. Since added H_2O_2 did not increase the rate of hydroxylation, enough must have been generated in the reaction mixture.

The slight inhibitory effect of superoxide dismutase on dihydroxyfumarate oxidation suggests that $\text{O}_2^{\cdot-}$ is involved in part of this process, in agreement with the results reported by Yamazaki & Yamazaki (1973) and Rotilio *et al.* (1975). The non-enzymic breakdown of $\text{O}_2^{\cdot-}$ could help to generate the H_2O_2 required (eqn. 1):

The complete inhibition by dismutase shows that $\text{O}_2^{\cdot-}$ is essential for hydroxylation [the claim by Paschen & Weser (1973) that superoxide dismutase is not specific for the breakdown of $\text{O}_2^{\cdot-}$ has been shown to be invalid; see Matheson *et al.* (1975)]. Cu^{2+} , which reacts with $\text{O}_2^{\cdot-}$ under these conditions (Fig. 1), had very similar effects to those of dismutase on hydroxylation and dihydroxyfumarate oxidation (Table 1). Mn^{2+} , which also reacts with $\text{O}_2^{\cdot-}$ (Fig. 1), inhibited hydroxylation but actually increased the rate of dihydroxyfumarate oxidation. Mn^{2+} reacts with $\text{O}_2^{\cdot-}$ to give Mn^{3+} (Lumsden & Hall, 1975), which probably acts as a catalyst of dihydroxyfumarate oxidation and changes the mechanism of the reaction (Yamazaki & Piette, 1963). Neither Mn^{2+} nor Cu^{2+} significantly inhibited hydroxylation by the Fe^{2+} /dihydroxyfumarate system or the $\text{EDTA}/\text{Fe}^{2+}$ /ascorbate system, which were almost insensitive to superoxide dismutase.

$\text{O}_2^{\cdot-}$ can react with H_2O_2 in solution to give the hydroxyl radical $\cdot\text{OH}$ (Haber & Weiss, 1934; Beauchamp & Fridovich, 1970). $\cdot\text{OH}$ is a powerful and unspecific hydroxylating agent, which would account for the wide range of aromatic compounds hydroxylated by the peroxidase/dihydroxyfumarate system. Its involvement is indicated by the inhibition of hydroxylation by scavengers of $\cdot\text{OH}$; this inhibition could not be attributed to an effect on dihydroxyfumarate oxidation. High concentrations of scavengers had to be added to achieve inhibition of hydroxylation, probably because $\cdot\text{OH}$ radicals react much faster with aromatic compounds than with the scavengers used (Beauchamp & Fridovich, 1970).

The results in Table 1 suggest that at least 50% of the *p*-coumarate hydroxylation is due to the action of $\cdot\text{OH}$ radicals. Since $\text{O}_2^{\cdot-}$ is essential for hydroxylation, this might mean that some $\text{O}_2^{\cdot-}$ brings about hydroxylation by a mechanism that does not involve $\cdot\text{OH}$. On the other hand, the concentrations of $\cdot\text{OH}$

scavengers might simply not be high enough to intercept all the $\cdot\text{OH}$ formed.

What is the physiological significance of hydroxylation of *p*-coumaric acid by this system? High concentrations of dihydroxyfumarate are not present in plants. Naturally occurring products, such as ascorbate, H_2O_2 (Halliwell, 1974), cysteine or NADH, could not replace it. Unless the reaction can utilize some other compound found naturally, it seems that this hydroxylation is unlikely to occur *in vivo*. However, it is of interest because it shows the role of $\text{O}_2^{\cdot-}$ in the mechanism of reactions catalysed by horseradish peroxidase.

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