The Action of Hydrogen Peroxide on the Hydroxylation of *p*-Coumaric Acid by Spinach-Beet Phenolase

By PETER F. T. VAUGHAN and ROSEMARY J. MCINTYRE*

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, U.K.

(Received 22 September 1975)

Treatment of spinach-beet phenolase with H_2O_2 under aerobic conditions results in a stimulation of the *p*-coumaric acid hydroxylation it catalyses, but not the caffeic acid oxidation. Spectroscopic evidence suggests that an oxygenated enzyme species is formed under these conditions.

The hydroxylation of *p*-coumaric acid to caffeic acid is catalysed by a phenolase purified from spinach-beet (*Beta vulgaris*, L.) leaves which has the stoichiometry of a mixed-function oxidase (Vaughan & Butt, 1969). Kinetic studies (McIntyre & Vaughan, 1975) are consistent with a mechanism in which an oxygenated enzyme species occurs as an intermediate in the catalytic cycle. The present communication describes two effects of H_2O_2 in the presence of air on spinach-beet phenolase: (1) production of an enzyme species with spectral properties similar to mushroom oxytyrosinase (Jolley *et al.*, 1974) and (2) the stimulation of *p*-coumaric acid hydroxylation, but not catechol oxidation, by the enzyme.

Materials and methods

Materials. H_2O_2 (100-vol.; AnalaR grade; BDH, Poole, Dorset, U.K.) was standardized spectrophotometrically by using $\epsilon_{240} = 36.0 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Biochemica Catalogue, C. F. Boehringer und Soehne G.m.b.H., Mannheim, West Germany). All other fine chemicals and definition of enzyme units have been described previously (Vaughan & Butt, 1969; McIntyre & Vaughan, 1975). Phenolase was prepared by the modified method of Vaughan & Butt (1970) and stored as described by McIntyre & Vaughan (1975).

Enzyme assays. Hydroxylation, in the presence of electron donors, was followed by measuring, spectrophotometrically, the quantity of caffeic acid produced during aerobic incubation at 30°C (McIntyre & Vaughan, 1975). Hydroxylation in the absence of electron donor was followed by measuring the increase in E_{420} due to the oxidation of caffeic acid, catalysed by the catechol oxidase activity of phenolase. A relationship between the yellow product of caffeic acid oxidation and the molar extinction coefficient of caffeic acid was obtained by adding

enzyme to caffeic acid ($0.1 \mu\text{mol}$) under conditions for catechol oxidase assay in the absence of electron donor. The reaction was followed at 420 nm until no change in extinction was observed, subsequent to the addition of further amounts of enzyme or air. The molar extinction coefficient for the oxidation product of caffeic acid (referred to as caffeic acid equivalent) at 420 nm was $3.18 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ under these conditions.

Catechol oxidase activity was followed by measuring the oxidation of caffeic acid aerobically at 30°C in a 10 mm quartz cuvette by a modification of the method of Sisler & Evans (1958). The enzyme was added to caffeic acid ($0.2 \mu\text{mol}$), $(\text{NH}_4)_2\text{SO}_4$ (1.5 mmol), Na_2HPO_4 ($100 \mu\text{mol}$)–citric acid ($40 \mu\text{mol}$) buffer, $\text{pH } 6.5$, in the absence or in the presence of ascorbate ($0.5 \mu\text{mol}$). The reaction was followed either by measuring the increase in E_{420} due to formation of caffeic acid oxidation products in the absence of electron donor, or by measuring the decrease in E_{265} due to the oxidation of ascorbate linked to reduction of caffeoyl-*o*-quinone.

Protein concentration was determined by using an $E_{1\text{cm}}^{1\%}$ (276 nm) value of 0.35 (Vaughan *et al.*, 1975).

Difference spectroscopy. Enzyme (2 mg) in 0.1 M Na_2HPO_4 – 0.05 M –citric acid buffer, $\text{pH } 6.5$, total volume 1.0 ml , was placed in both sample and reference cuvette in a Cary 15 recording spectrophotometer. H_2O_2 (80 nmol) was added to the sample cuvette, and a corresponding volume of water to the reference cuvette, and the resulting spectrum was measured over the range 400 – 280 nm with 0.1 absorbance full-scale deflexion. Immediately after the recording of the spectrum (about 10 min), catalase (250 units) was added to both cuvettes and the spectrum was redetermined.

Chromatography of reaction mixture. *p*-Coumaric acid ($10 \mu\text{mol}$) was incubated with enzyme (150 m-units), Na_2HPO_4 ($100 \mu\text{mol}$)–citric acid ($40 \mu\text{mol}$) buffer, $\text{pH } 6.5$, $(\text{NH}_4)_2\text{SO}_4$ (1.5 mmol) and H_2O_2 ($16 \mu\text{mol}$) in the absence and in the presence of ascorbate ($10 \mu\text{mol}$) under the conditions used by Vaughan & Butt (1969). The reaction was terminated

* Present address: Development and Metabolic Neurology Branch, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Md. 20014, U.S.A.

after 7.5 min by addition of 5M-H₂SO₄ (1.0 ml) to each flask, and the acidified reaction mixture was extracted three times with diethyl ether (5.0 ml). The combined ether extracts were evaporated to dryness, the residue was taken up in ethanol (0.1 ml), and 10 μ l portions were spotted on to Whatman no. 1 chromatography paper. Chromatograms were developed in the upper layer of benzene-acetic acid-water (6:7:3, by vol.) solvent system (Ibrahim & Towers, 1960). Chromatograms were examined under a u.v. lamp after being sprayed with diazotized sulphanilic acid. Spots were identified by position, u.v. fluorescence or absorption and colour compared with *p*-coumaric acid and caffeic acid markers. Control reactions that did not contain H₂O₂ were treated in the same way.

Results and discussion

Incubation of 96 munits of enzyme with 1.67 mM-*p*-coumaric acid, at pH 5.3 for 70 min, in the absence of electron donor resulted in the formation of yellow oxidation product, equivalent to the complete oxidation of 0.12 μ mol of caffeic acid. This is in contrast with a previous report (Vaughan & Butt, 1969) in which no consumption of *p*-coumaric acid was detected in the absence of electron donors. The probable reason for this discrepancy is the greater sensitivity of the spectrophotometric assay; measurement of consumption of 0.12 μ mol of *p*-coumaric acid against a blank value of 10 μ mol would have been difficult with the colorimetric assay used in the previous study.

Effect of H₂O₂ on the progress of hydroxylation. Incubation of *p*-coumaric acid and enzyme, with up to 0.267 mM-H₂O₂, in the absence of electron donor, decreased the lag period in hydroxylation from 200 s to 20 s (Fig. 1a). The lag period is measured by extrapolating the linear portion of the time-course to the abscissa and measuring the displacement of the intercept from the origin. The decrease in lag time was accompanied by a twofold increase in hydroxylation rate. A tenfold stimulation of hydroxylation to 101.6 μ mol of caffeic acid equivalents/min per mg of protein was observed with 13.3 mM-H₂O₂. In the presence of ascorbate the lag period was eliminated by 1.33 mM-H₂O₂, whereas 13.3 mM-H₂O₂ resulted in a fivefold stimulation of hydroxylation to 84.0 μ mol of caffeic acid/min per mg of protein (Fig. 1b). With NADH and dimethyltetrahydropteridine (2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine) as electron donors the lag period was eliminated by 2.0 mM-H₂O₂. No stimulation of hydroxylation was observed with up to 13.3 mM-H₂O₂ in the presence of dimethyltetrahydropteridine, whereas with NADH the addition of 6.67 mM-H₂O₂ resulted in a fourfold stimulation of caffeic acid production.

Bordner & Nelson (1939) found that H₂O₂ shortened the initial lag in the aerobic oxidation of

Fig. 1. Effect of H₂O₂ on the progress of hydroxylation

(a) Caffeic acid equivalents production rate (●) and lag time (○) when 144 munits of enzyme (Stage IV; Vaughan & Butt, 1970) were incubated with *p*-coumaric acid (5 μ mol) and the amounts of H₂O₂ indicated in 0.1M-Na₂HPO₄-0.05M-citric acid buffer, pH 6.5, containing (NH₄)₂SO₄ (1.5 mmol) in a total volume of 3 ml at 30°C in air. (b) Caffeic acid production (●) and lag time (○) when 52 munits of enzyme were incubated with *p*-coumaric acid (5 μ mol), ascorbate (10 μ mol) and EDTA (10 μ mol) under the conditions described in (a). The values in parentheses on the ordinate represent the hydroxylation rate and lag time in the absence of peroxide.

p-cresol by mushroom tyrosinase. They suggested that H₂O₂ was acting by reducing *o*-quinones to *o*-dihydric phenols, which in turn shortened the lag period. This mechanism is unlikely to operate with the spinach-beet enzyme because a lag period is observed in the presence of electron donors, i.e. under conditions in which *o*-dihydric phenols would accumulate. The stimulation of hydroxylation in the presence of ascorbate provides additional evidence against the view that H₂O₂ acts by reducing caffeoyl-*o*-quinone to caffeic acid. Thus caffeic acid, although eliminating the lag period observed in the presence of ascorbate, did not stimulate hydroxylation of *p*-coumaric acid (McIntyre & Vaughan, 1975).

Comparison between reaction product in the presence and in the absence of H₂O₂. When *p*-coumaric acid was incubated with enzyme, under the standard conditions of assay in the absence of electron donor, the resulting spectrum showed an increase in absorbance over the range 520 to 340 nm, with a plateau between 420 and 380 nm. When ascorbate was included in the reaction mixture a single peak, with absorption maximum at 340 nm, was observed. In both cases the reference cuvette contained the complete reaction

Fig. 2. Effect of catalase on the difference spectrum of H_2O_2 -treated phenolase

Enzyme (2mg) in 0.1M- Na_2HPO_4 -0.05M-citric acid buffer, pH6.5, total volume 1.0ml (○), was treated with 80nmol of H_2O_2 (●), followed by catalase (250units) (Δ). Each spectrum was measured against the same concentration of untreated enzyme in the reference cuvette.

mixture without the addition of enzyme. Inclusion of 3.33mM- H_2O_2 did not alter either of these spectra, despite the tenfold increase in hydroxylation in the absence of electron donor (Fig. 1a) or threefold increase with ascorbic acid (Fig. 1b) observed with this concentration of H_2O_2 . Further, chromatography of the reaction mixture showed that caffeic acid was the only product in the presence of 3.33mM-ascorbate. In the absence of electron donor the intensity of the caffeic acid spot was greatly diminished and a yellowish-green fluorescent streak was apparent at the origin. The presence of 5.33mM- H_2O_2 did not alter the chromatographic pattern observed either in the presence or in the absence of ascorbic acid.

The similarity between the product of *p*-coumaric acid hydroxylation, in the presence and in the absence of H_2O_2 , whether identified spectrophotometrically or chromatographically, suggests that the increase in absorbance observed with H_2O_2 represents a stimulation of the hydroxylase activity of phenolase, rather than an artifact due to reaction between H_2O_2 and caffeoyl-*o*-quinone, similar to the reaction between H_2O_2 and *o*-quinones reported by Doskočil (1950). This conclusion was supported by the observation that generation of caffeoyl-*o*-quinone non-enzymically by 0.26mM- $K_3Fe(CN)_6$ in the presence of 1.67mM-*p*-coumaric acid, 0.017mM-caffeic acid and 13.3mM- H_2O_2 did not lead to an increase in E_{420} . Further,

3.33mM- H_2O_2 had no effect on catechol oxidase activity either in the presence or in the absence of ascorbate.

These results suggest that H_2O_2 stimulates the hydroxylase activity of spinach-beet phenolase but not the catechol oxidase activity. This is similar to the observations made by Pugh (1930), who found that addition of H_2O_2 to meal-worm tyrosinase accelerated colour formation from monophenols but not from *o*-dihydric phenols.

More direct evidence for a reaction between H_2O_2 and spinach-beet phenolase is provided by the formation of an enzyme species with absorption maximum at 345 nm, which is reversed by catalase (Fig. 2). This is similar to the reaction between mushroom phenolase and H_2O_2 reported to Jolley *et al.* (1972). Evidence that this species is an intermediate, in the mushroom phenolase catalytic cycle, is provided by its rapid decay when allowed to react with monophenol or *o*-dihydric phenol substrates (Makino & Mason, 1973). The rapid formation of product, absorbing at 340 nm, when 50 nmol of *p*-coumaric acid was added to spinach-beet phenolase, treated with H_2O_2 , prevented a similar analysis on the effect of substrate on the enzyme species. However, in contrast with spinach-beet phenolase, no stimulation of the overall rate of mushroom phenolase activity was observed.

It is suggested, from this present study, that there is a reaction between H_2O_2 and spinach-beet phenolase, similar to that observed with the mushroom enzyme, to form an oxygenated enzyme species effective in the hydroxylation of *p*-coumaric acid. Thus additional support is provided for the occurrence of an oxygenated enzyme species, postulated previously (McIntyre & Vaughan, 1975) from kinetic studies on the hydroxylation of *p*-coumaric acid, and that the formation of this oxygenated complex is a rate-limiting step under the conditions employed.

We thank Professor R. M. S. Smellie for providing facilities to carry out this work, Mr. A. Wilson for his skilled technical assistance and the Science Research Council for the award of a Research Studentship to R. J. McL.

- Bordner, C. A. & Nelson, J. M. (1939) *J. Am. Chem. Soc.* **61**, 1507-1513
 Doskočil, J. (1950) *Collect. Czech. Chem. Commun.* **15**, 614-629
 Ibrahim, R. K. & Towers, G. H. N. (1960) *Arch. Biochem. Biophys.* **87**, 125-128
 Jolley, R. L., Evans, L. H. & Mason, H. S. (1972) *Biochem. Biophys. Res. Commun.* **46**, 878-884
 Jolley, R. L., Evans, L. H., Makino, N. & Mason, H. S. (1974) *J. Biol. Chem.* **249**, 335-345
 Makino, N. & Mason, H. S. (1973) *J. Biol. Chem.* **248**, 5731-5735

- McIntyre, R. J. & Vaughan, P. F. T. (1975) *Biochem. J.* **149**, 447-461
- Pugh, M. (1930) *Biochem. J.* **24**, 1442-1455
- Sisler, E. C. & Evans, H. J. (1958) *Plant Physiol.* **33**, 255-257
- Vaughan, P. F. T. & Butt, V. S. (1969) *Biochem. J.* **113**, 100-115
- Vaughan, P. F. T. & Butt, V. S. (1970) *Biochem. J.* **119**, 89-94
- Vaughan, P. F. T., Eason, R., Paton, J. Y. & Ritchie, G. A. (1975) *Phytochemistry* **14**, in the press