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Conversion of *p*-Coumarate into Caffeate by *Streptomyces nigrifaciens*

PURIFICATION AND PROPERTIES OF THE HYDROXYLATING ENZYME

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1. An enzyme responsible for the conversion of *p*-coumarate into caffeate was purified 97-fold from *Streptomyces nigrifaciens*. The enzyme had a molecular weight of 18000 as determined by Sephadex G-100 gel filtration and was homogeneous on polyacrylamide-gel electrophoresis. 2. The preparation exhibited both *p*-coumarate hydroxylase and caffeate oxidase activities. 3. Stoicheiometry of the reaction indicated a mono-oxygenase-mediated catalysis consuming 1 mol of O_2/mol of substrate hydroxylated. 4. NADH, NADPH, tetrahydropteroylglutamate or ascorbate act as electron donors for the reaction, ascorbate being inhibitory at higher concentrations. 5. The optimum enzyme activity was at about pH 7.7 and 40°C, with an activation energy of 39 kJ/mol. 6. Monophenols such as *p*-hydroxyphenylpropionate, *p*-hydroxyphenylacetate, L-tyrosine and DL-*p*-hydroxyphenyl-lactate were also hydroxylated by the preparation, in addition to *p*-coumarate. 7. The enzyme was a copper protein having 0.38% copper in a bound form. 8. Thiol-group inhibitors did not affect the reaction. 9. The relationship of the enzyme to other hydroxylases is discussed.

The metabolism of aromatic compounds is often initiated by hydroxylation, since these compounds are generally oxygen deficient and need to be oxygenated to become biologically active or more soluble in water (Hayaishi, 1969). Many micro-organisms, particularly saprophytic ones, have been reported to utilize a great variety of naturally occurring aromatic compounds for their growth, but only a few enzymes concerned with the hydroxylation of the aromatic ring have been characterized. Those characterized include phenylalanine hydroxylase (Guroff & Ito, 1963), melilotate hydroxylase (Levy, 1967), salicylate hydroxylase (Yamamoto *et al.*, 1965), *m*-hydroxybenzoate hydroxylase (Premakumar *et al.*, 1969) and *p*-hydroxybenzoate hydroxylase (Hosokawa & Stanier, 1966). No enzyme isolated from any micro-organism, however, is known to hydroxylate *p*-coumarate. In the present paper we report the purification and properties of an enzyme from *Streptomyces nigrifaciens* that can hydroxylate *p*-coumarate and related phenolic acids substituted in the *para* position.

Enzyme preparations from higher plants (Sato, 1966; Vaughan & Butt, 1969*a*,*b*) and mushroom (Sato, 1969) catalyse hydroxylation of *p*-coumarate and contain *o*-diphenol oxidase activity in addition to their ability to hydroxylate monophenols.

Such phenolases have also been found in bacteria and microfungi, although their purification has been achieved only from the fungal sources (Fling *et al.*, 1963; Herzfeld & Esser, 1969); most of the studies carried out on them have been confined to the physical properties and the *o*-diphenol oxidase activity of the enzyme. In addition, interpretation of the results on the hydroxylase activity of the enzyme is made difficult because of the dual functions of these enzymes. Ascorbate, which is usually employed as a reductant in the hydroxylation, also reduces the *o*-quinone subsequently produced, thus facilitating the accumulation of the dihydroxyphenol in the medium. Vaughan & Butt (1969*b*) were able to measure the hydroxylase activity of their spinach preparation by providing excess of reductant to prevent the further oxidation of caffeate. The *Streptomyces* enzyme was also found to contain diphenol oxidase activity, which is characteristic of a typical phenolase. It was, however, possible for us to study the hydroxylase reaction selectively, since caffeate oxidase activity could be completely inhibited by *p*-coumarate, the substrate for the hydroxylation step.

Materials and Methods

Materials

Chemicals. Alcohol dehydrogenase (from yeast), *p*-hydroxymercuribenzoate, NADPH, NADH and H_4PteGlu* were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Mercaptoethanol, GSH and ascorbic acid were supplied by British Drug

*Abbreviation: H_4PteGlu, tetrahydropteroylglutamate.

Houses Ltd., Poole, Dorset, U.K., and dithiothreitol was a gift from P-L Biochemicals, Milwaukee, Wis., U.S.A. The aromatic chemicals were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and recrystallized before use and Sephadex G-100 was from Pharmacia, Uppsala, Sweden. All other chemicals used were analytical grades available commercially.

Organism. A number of species of *Streptomyces* were isolated from soil by enrichment culture by using *p*-coumarate as sole source of carbon. The organism used throughout the present work was identified as *Streptomyces nigrifaciens.*

Methods

U.v. and i.r. spectroscopy. The u.v. spectra were recorded on a Unicam SP.500 spectrophotometer. A Perkin-Elmer (Infracord) model 137 spectrometer was used to determine the i.r. spectra of the synthetic and isolated enzymic products (in a Nujol phase).

Medium and conditions of growth. S. nigrifaciens was grown on a nutrient medium containing malt extract (3%), peptone (0.5%) and yeast extract (0.1%). The pH of the medium was adjusted to 7.0 with 1M-NaOH. The flasks (500ml) containing 100ml of medium were autoclaved for 10min at 120°C and inoculated with a heavy suspension of spores. For preparation of cell extracts the cells were harvested by filtering through Terylene cloth and were washed several times with water. Fresh cells were always used for enzyme preparation.

Enzyme assays. Enzyme assays were done at 30°C. One unit of enzyme activity is defined as the amount that catalyses the transformation of 1μmol of the substrate or the formation of 1μmol of product/min under the conditions of the assay. Specific activity is expressed as milliunits of the enzyme/mg of protein.

Measurement of hydroxylase activity. The hydroxylase activity was measured by incubating a reaction mixture (1ml) containing tris-HCl buffer, pH7.7 (30μmol), *p*-coumaric acid (2μmol), NADH (2μmol) and 0.2ml of enzyme (15-20 milliunits) at 30°C for 15min. The reaction was stopped by the addition of 1ml of 0.5M-HCl, and the caffeate formed was determined by the method described by Arnow (1937) for the measurement of 3,4-dihydroxyphenylalanine (dopa).

Assay of o-diphenol oxidase activity. A reaction mixture (1ml) consisting of tris-HCl buffer, pH7.7 (30μmol), caffeic acid (0.25μmol) and the enzyme preparation (15-20 milliunits) was incubated for 15min at 30°C. After the reaction had been stopped with 1ml of 0.5M-HCl, the disappearance of caffeate was determined colorimetrically (Arnow, 1937).

O_2 consumption. The conventional Warburg technique (Umbreit *et al.*, 1964) was used to measure O_2 consumption during the hydroxylation reactions. O_2 consumption was also measured with the Gilson model K Oxygraph to establish stoicheiometry.

Determination of protein. The method of Lowry *et al.* (1951) was used for the determination of protein. Dry bovine serum albumin was the standard.

Determination of copper. The copper content of the purified enzyme was determined by the method of Stark & Dawson (1958).

Determination of molecular weight. The molecular weight of the enzyme was determined by the method of Andrews (1964) by filtering the protein through a calibrated column (1.5cm×40cm) of Sephadex G-100 with a flow rate of 18ml/h. The column was equilibrated with 25mM-sodium phosphate buffer (pH7); the purified enzyme (3mg of protein) was loaded on to the column and 2ml fractions were collected. The standard proteins used were cytochrome *c* (mol.wt. 12500), pepsin (mol.wt. 35500), hexokinase (mol.wt. 47000) and bovine serum albumin fraction V (mol.wt. 60000).

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was done essentially by the method of Davis (1964) with glycine-tris buffer, pH8.6, and L-alanine buffer, pH4.3. Electrophoresis was for 40min at 5°C with a current of 6mA/tube. The gel, after removal from the tube, was stained for 45min with Amido Black and de-stained in 7% (w/v) acetic acid.

Purification of the enzyme. All operations were done at 0-4°C.

Step I: crude extract. Washed cells of *S. nigrifaciens* (20g) were suspended in 60ml of 50mM-sodium phosphate buffer, pH7.0. Batches (30ml) were treated for 10min in a 10kHz Raytheon sonic oscillator cooled with circulating iced water and then they were centrifuged for 20min at 8000*g*. The clear supernatant (80ml), having a specific activity of 3-6 milliunits/mg of protein, was used as crude extract.

Step II: protamine sulphate treatment. The supernatant solution from step I (76.5ml) was treated dropwise with 0.1 vol. of a 2% (w/v) solution of protamine sulphate dissolved in 25mM-sodium phosphate buffer, pH7.0 (8.5ml). After being stirred gently for 10min, the suspension was centrifuged at 12000*g* for 10min and the precipitate was discarded. This procedure removed a large amount of inactive protein and material absorbing at 260nm.

Step III: first ammonium sulphate fractionation. To the above supernatant (75ml) solid $(NH_4)_2SO_4$ (18.4g) was added with stirring to bring the solution to 40% saturation. The pH was maintained at 7 with a dilute solution of NH_3. After the mixture had stood for 20min in an ice bath the precipitate was removed by centrifugation and discarded. The concentration of the supernatant fluid was raised to 70% saturation by a further addition of solid $(NH_4)_2SO_4$ (15.4g). After the mixture had stood for 1h the resulting precipitate was collected by centrifugation, dissolved

in the minimum volume of 25mM-sodium phosphate buffer, pH7.0 (8ml), and desalted by passage through a column (2cm×26.4cm) of Sephadex G-25 (bead form) that had been previously equilibrated with 25mM-sodium phosphate buffer, pH7.0. The active protein fraction was collected in 17ml of effluent after the void volume (36ml).

Step IV: DEAE-cellulose treatment. The enzyme responsible for the hydroxylation of *p*-coumarate was not adsorbed on DEAE-cellulose under the experimental conditions used. Hence the following rapid procedure involving DEAE-cellulose was used for the removal of some inert protein.

DEAE-cellulose was washed by the method of Peterson & Sober (1962) and suspended in 25mM-sodium phosphate buffer, pH7.0. A portion (20ml) of the suspension was filtered on a Buchner funnel. The enzyme preparation obtained after passage through the Sephadex G-25 column (17ml) was added to washed DEAE-cellulose cake (1g of DEAE-cellulose/15ml of column effluent) and the mixture was stirred mechanically for 30min. The suspension was filtered on a Buchner funnel and the filtrate was collected.

Step V: second ammonium sulphate precipitation. To the DEAE-cellulose supernatant (17ml) was added solid $(NH_4)_2SO_4$ (9.9g) with mechanical stirring to give 80% saturation. The mixture was equilibrated for 1h. The resulting precipitate was collected by centrifuging at 10000*g* for 15min and dissolved in 25mM-sodium phosphate buffer, pH7.0 (8ml).

Step VI: Sephadex G-100 filtration. A 5ml portion of the concentrated enzyme from step V was chromatographed on a column (2cm×20cm) of Sephadex G-100. The column was eluted with 25mM-sodium phosphate buffer, pH7.0, at an average flow rate of 30ml/h. Fractions (4ml) were collected and assayed for *p*-coumarate hydroxylase activity. The elution pattern of the enzyme from Sephadex G-100 is shown in Fig. 1. The active fractions were colourless.

The enzyme preparations at various stages of

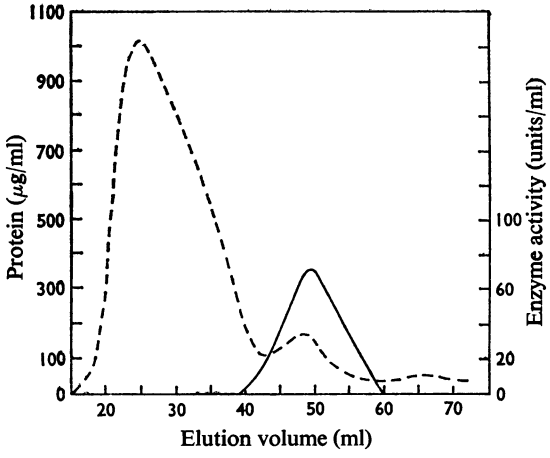

Fig. 1. *Sephadex G-100 gel elution pattern of the p-coumarate hydroxylating enzyme from S. nigrifaciens*

Details are given in the text. ----, Protein; ——, enzyme activity

Table 1. *Purification of the p-coumarate hydroxylating system from S. nigrifaciens*

p-Coumarate hydroxylase activity was measured by incubating, for 15min at 30°C, a reaction mixture (1ml) containing tris–HCl buffer, pH7.7 (30μmol), *p*-coumaric acid (2μmol), NADH (2μmol), ethanol (2.5μmol), alcohol dehydrogenase (100 milliunits) and a suitable amount of enzyme. Other conditions for the assay of *p*-coumarate hydroxylase activity as well as the conditions for determination of caffeate oxidase activity were as described in the Materials and Methods section.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (milliunits)		Specific activity (milliunits/mg of protein)		Hydroxylase/ oxidase activity ratio
			p-Coumarate hydroxylase	Caffeate oxidase	*p*-Coumarate hydroxylase	Caffeate oxidase	
I: crude extract	76	425.6	2026	1368	4.7	3.2	1.49
II: protamine sulphate	75	217.5	2700	1795	12.4	8.2	1.39
III: first $(NH_4)_2SO_4$ (40–70% satn.)	17	72.4	2380	1593	32.9	22.0	1.45
IV: DEAE-cellulose	17	51.0	3026	1851	59.5	36.3	1.64
V: second $(NH_4)_2SO_4$ (0–80% satn.)	8	36.8	2453	1634	66.6	44.5	1.50
VI: Sephadex G-100	4	0.84	360	240	428.5	285.6	1.50

purification were found to have both *p*-coumarate hydroxylase (monophenol oxidase) and caffeate oxidase (diphenol oxidase) activities. Table 1 summarizes the yields and specific activities of hydroxylase and diphenol oxidase activities for each step of the purification procedure. An overall yield of 18% through step I to step VI was achieved with 96- and 89-fold increases in the specific activities of *p*-coumarate hydroxylase and caffeate oxidase activities respectively. The ratio of hydroxylase activity to caffeate oxidase activity was more-or-less constant throughout the purification.

Isolation of the product of p-coumaric acid hydroxylation. A large-scale incubation mixture (200ml) consisting of sodium phosphate buffer, pH7.7 (7mmol), *p*-coumaric acid (0.8mmol), ascorbic acid (0.4mmol) and purified enzyme from step VI (40ml) was incubated at 30°C with occasional stirring. At 15min intervals 0.1 M-ascorbic acid (1ml each time) was added. After 2h the pH of the reaction mixture was adjusted to 2 with 1M-HCl and it was extracted twice with equal volumes of peroxide-free ether. The ethereal layer was evaporated to dryness after being shaken with anhydrous Na_2SO_4. The procedure adopted for the isolation of caffeic acid from the residue was essentially similar to that described by Sato (1969). The crude product was dissolved in 0.2M-sodium acetate (25ml). After the addition of 0.2M-EDTA (0.5ml) and alumina (5g) to the solution the pH was adjusted to 8.5 by dropwise addition of 2M-NH_3, then the suspension was stirred for 5min. The slurry was transferred to a column and washed with distilled water (100ml). This process removed *p*-coumaric acid, whereas caffeic acid was retained on the column. Subsequent elution of the column with 0.6M-acetic acid (30ml) followed by extraction of the eluate with peroxide-free ether resulted in the transfer of caffeic acid into the ether phase. The organic layer was shaken with anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in the minimum volume of hot water and recrystallized twice to yield 20mg of the product. Both authentic caffeic acid and the isolated product melted at 195°C and there was no change in melting point on admixture. The i.r. and u.v. spectra of the product and the authentic compound were also identical.

Results

Properties of the purified p-coumarate-hydroxylating enzyme

Homogeneity. The enzyme was found to yield only one protein band on disc electrophoresis at both alkaline pH (8.6) and acid pH (4.3).

Inhibition of caffeate oxidase activity of the purified enzyme by p-coumarate. Since electrophoretically homogeneous enzyme preparations obtained from *Streptomyces* exhibited both *p*-coumarate hydroxylase and caffeate oxidase activities, attempts were made

Table 2. *Effect of various monophenols on the caffeate oxidase activity of the purified Streptomyces enzyme*

Purified *Streptomyces* enzyme was preincubated with each of the compounds, except the *p*-hydroxymonophenols, for 10min before the addition of caffeic acid as substrate. The *o*-diphenol oxidase activity was measured as described in the Materials and Methods section. The following monophenols had no effect on caffeate oxidation at concentrations ranging from 0.5 to 1.0mM: *p*-hydroxymandelate, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoate, *o*-coumarate, *o*-hydroxyphenylacetate, salicylate, *m*-hydroxymandelate, *m*-coumarate, *m*-hydroxybenzoate and *trans*-cinnamate.

Substrate	Concn. (mM)	Inhibition (%)
None	—	0
p-Hydroxyphenylacetate	0.5	21
	1.0	39
Benzoate	0.5	0
	1.0	2
L-Tyrosine	0.5	10
	1.0	15
p-Coumarate	0.5	79
	1.0	100

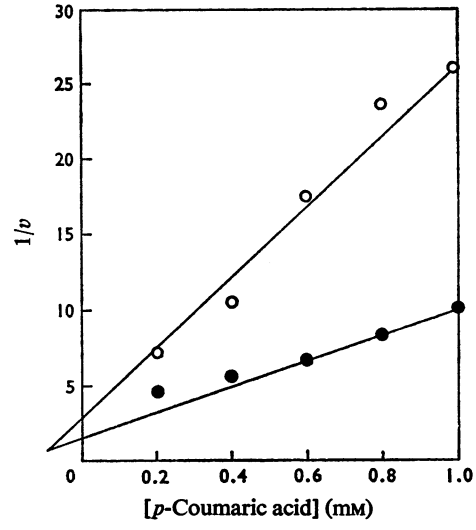

Fig. 2. *Dixon plot for caffeate oxidase activity of the purified Streptomyces enzyme*

The velocity of the reaction was determined at two fixed concentrations of caffeate: ○, 0.25mM; ●, 0.5mM. Details are given in the text.

to inhibit the second reaction selectively. The effect of various structural analogues on caffeate disappearance in the presence of purified enzyme is summarized in Table 2. *p*-Coumarate was the most potent inhibitor, and it completely suppressed the oxidase activity at a concentration of 1.5mM. The inhibition, as shown in Fig. 2 was competitive. Because of this interesting property of the enzyme it was consequently possible to study the *p*-coumarate hydroxylase reaction without interference from the *o*-diphenol oxidase oxidizing the caffeate formed. Complete inhibition of the *o*-diphenol oxidase reaction was ensured by adding 2mM-*p*-coumarate to the standard reaction mixture.

Stability. The purified enzyme from step VI in 25mM-sodium phosphate buffer, pH7 (0.2–0.25mg of protein/ml) lost 89% of its activity in 72h on storage at 4°C. Freezing of the enzyme at −20°C resulted in total loss of activity in 12h.

Molecular weight. The molecular weight of the *Streptomyces* enzyme possessing *p*-coumarate hydroxylase activity, as determined by gel filtration is approx. 18000.

U.v. spectrum. The purified enzyme gave a u.v. spectrum characteristic of a simple protein without showing any apparent bound flavin prosthetic group.

Substrate specificity. p-Coumarate was not the only monophenolic acid hydroxylated by the *Streptomyces* enzyme: *p*-hydroxyphenyl-lactate, *p*-hydroxyphenylpropionate, DL-*p*-hydroxyphenylacetate and L-tyrosine were also substrates (Table 3). Several other monophenols, including DL-*p*-hydroxymandelate, *p*-hydroxybenzoate, *p*-hydroxybenzaldehyde and *p*-hydroxybenzyl alcohol, were not hydroxylated. Monophenols having the hydroxyl group in the *ortho* or the *meta* position were not substrates for the purified hydroxylating enzyme.

Substrate specificity for reductant. There was no detectable hydroxylase activity when the purified enzyme was incubated with the substrate in the absence of any cofactors under standard assay conditions. Addition of various reductants, however,

Table 3. *Specificity of the purified p-coumarate hydroxylating enzyme from S. nigrifaciens for various monophenols*

O_2 uptake was measured by using the conventional Warburg technique. Each flask contained, in a total volume of 3.2ml, tris–HCl buffer (90μmol), 0.6ml of enzyme (from step VI), 6μmol of NADH and 6μmol of substrate in the side arm. The central well contained 0.2ml of 20% (w/v) KOH. The substrate was tipped at zero time and incubation was at 30°C in an atmosphere of air.

Substrate	Relative activity
p-Coumarate	100
p-Hydroxyphenylpropionate	170
p-Hydroxyphenylacetate	68
L-Tyrosine	59
DL-*p*-Hydroxyphenyl-lactate	40

Table 4. *Effect of various reductants on the hydroxylation of p-coumarate and p-hydroxyphenylacetate by the purified hydroxylating enzyme from S. nigrifaciens*

Assay conditions were as given in the Materials and Methods section except that different reductants were used at the stated concentrations. Homoprotocatechuate was the product formed from *p*-hydroxyphenylacetate. Its formation was studied in the same way as was that of caffeate.

Reductant	Concn. (mM)	Caffeate formed (nmol)	Homoprotocatechuate formed (nmol)
NADH	0.5	185	91
	1.0	220	124
	1.5	220	148
	2.0	220	148
NADPH	0.5	200	17
	1.0	220	17
	1.5	220	26
	2.0	215	26
Ascorbate	0.5	135	180
	1.0	175	190
	1.5	160	200
	2.0	120	200
H_4PteGlu	0.5	180	75
	1.0	180	86
	1.5	180	75
	2.0	215	75

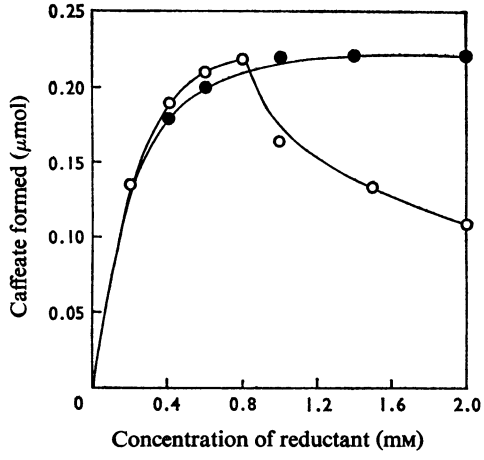

Fig. 3. *Effect of concentration of reductants on the hydroxylation of p-coumarate by the Streptomyces enzyme*

Conditions of assay were as given in the Materials and Methods section except that different concentrations of reductants were used. ●, NADH; ○, ascorbate.

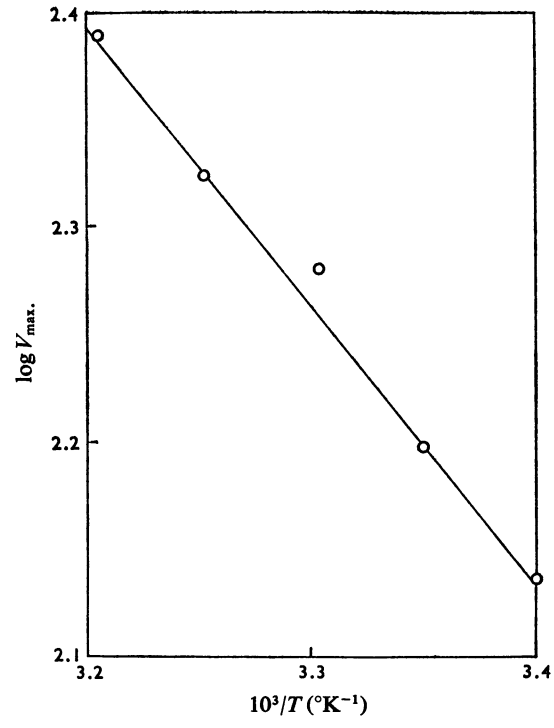

Fig. 4. *Arrhenius plot for the determination of the activation energy for the hydroxylation of p-coumarate by the Streptomyces enzyme*

For details see the Materials and Methods section.

considerably enhanced the rates of hydroxylation of *p*-coumarate and *p*-hydroxyphenylacetate (Table 4). NADH, NADPH, H_4PteGlu and ascorbate were all more-or-less equally effective in bringing about the enzymic hydroxylation of *p*-coumarate. Although ascorbate served as an electron donor in the reaction, at higher concentrations it inhibited caffeate formation (Fig. 3).

For the hydroxylation of *p*-hydroxyphenylacetate, however, ascorbate served as the best reductant, NADH, H_4PteGlu and NADPH being decreasingly less effective in that order. Unlike that of *p*-coumarate, the hydroxylation of *p*-hydroxyphenylacetate was not inhibited by increasing concentrations of ascorbate.

Effect of pH on the activity. The optimum rate of hydroxylation of *p*-coumarate in the presence of purified *Streptomyces* enzyme was at about pH7.7. However, the pH–activity curve with *p*-coumarate as substrate was sharp whereas that with *p*-hydroxyphenylacetate was rather broad.

Influence of temperature. Optimum hydroxylase activity was at about 40°C under the conditions employed. The activation energy for the reaction, calculated from the Arrhenius plot, is 39kJ/mol (Fig. 4).

Time-course of the reaction. The time-course of the *p*-coumarate hydroxylase reaction is shown in Fig. 5. In the presence of equimolar amounts of *p*-coumarate and NADH (2mM each) the reaction was more-or-less linear for up to 30min. However, when the concentration of the reductant was lowered to 0.33mM a sigmoid curve was obtained. This was due to an initial lag in the enzyme activity when NADH concentration was limiting. The reaction stopped after 30min and there was no apparent further accumulation of caffeate.

The rates of O_2 uptake in the presence and in the absence of NADH were followed in a Warburg respirometer. In the absence of NADH the O_2 uptake commenced only after 15min, indicating an initial lag in the absence of an electron donor.

Stoicheiometry of the reaction. The stoicheiometry of the *p*-coumarate hydroxylase reaction was established by correlating the amount of caffeate formed with that of O_2 consumed during the reaction. The standard reaction mixture was doubled (to 2ml) for study of the utilization of O_2 in a Gilson Oxygraph. Portions (0.5ml) from the reaction mixture were taken after 5, 10 and 15min of the reaction and diluted to 1ml with water for the determination of caffeate as described in the Materials and Methods section. The averaged results of four experiments,

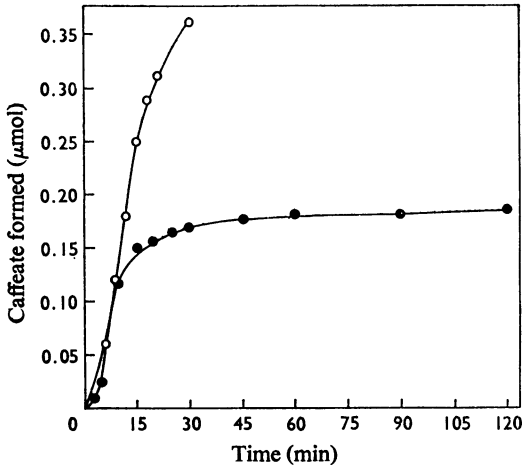

Fig. 5. *Time-course of caffeate formation in the presence of purified p-coumarate-hydroxylating enzyme from S. nigrifaciens*

Conditions of assay were as given in the Materials and Methods section except that two concentrations of NADH were used: o, 2mM; •, 0.33mM.

Table 5. *Stoicheiometry of the p-coumarate hydroxylase reaction*

O_2 consumption was measured in a Gilson Oxygraph model B. Samples were taken at different times and assayed for the formation of caffeate. Other conditions are given in the Materials and Methods section.

Time (min)	Caffeate formed (μmol)	O_2 consumed (μmol)
5	0.10	0.124
10	0.22	0.225
15	0.44	0.450

presented in Table 5, show that 1 mol of O_2 was consumed per mol of caffeate formed; this is in agreement with the stoicheiometry normally encountered in hydroxylation reactions catalysed by mono-oxygenases.

Reaction kinetics. A double-reciprocal plot of initial velocity against *p*-coumarate concentration was linear, indicating that Michaelis–Menten saturation kinetics are characteristic with the substrate. The calculated apparent K_m value was 0.37mM for *p*-coumarate.

Copper content. The *Streptomyces* enzyme possessing *p*-coumarate hydroxylase activity is a copper protein. Although colourless, the purified enzyme preparation contained 0.38% of copper, expressed as a percentage of the concentration of protein.

Table 6. *Effect of various inhibitors on the hydroxylation of p-coumarate by the purified Streptomyces enzyme*

For details see the text.

Inhibitor	Concn. (mM)	Inhibition (%)
None	—	0
αα′-Bipyridyl	0.1	0
	1.0	21
EDTA	0.1	5
	1.0	24
o-Phenanthroline	0.1	0
	1.0	44
Salicylaldoxime	0.1	0
	1.0	41
Diethyldithiocarbamate	0.1	70
	1.0	100
8-Hydroxyquinoline	0.1	11
	1.0	77
N-Ethylmaleimide	0.1	0
	1.0	0
p-Hydroxymercuribenzoate	0.1	0
	1.0	0

Effect of metal ions. A study of the effect of various metal ions showed that none of the bivalent cations tested, including Fe^{2+}, Cu^{2+}, Mn^{2+} and Hg^{2+} had either stimulatory or inhibitory effects on the formation of caffeic acid.

Inhibition studies. The effect of various inhibitors on the hydroxylation of *p*-coumarate is presented in Table 6. Even though the enzyme activity was not enhanced by any metal ions, the potent inhibition by metal-chelating agents such as *o*-phenanthroline, salicylaldoxime, diethyldithiocarbamate and 8-hydroxyquinoline suggests that a tightly bound metal ion, probably copper, is required for enzyme activity. The inhibition, however, was found to be irreversible, as neither Cu^{2+} nor any other metal ion could restore the activity.

Thiol-group inhibitors such as *N*-ethylmaleimide and *p*-hydroxymercuribenzoate had no effect on the reaction even at concentrations as high as 1mM.

Effect of thiol-reducing agents. Thiol-reducing agents such as GSH, 2-mercaptoethanol and dithiothreitol at concentrations of 0.01–0.1mM totally inhibited caffeate formation. Preincubation with Cu^{2+} or NADH did not prevent the inhibition, nor did Cu^{2+} reverse the inhibition.

Discussion

Hydroxylation of *p*-coumarate is a rare phenomenon in micro-organisms. One such reaction, observed by Power *et al.* (1965) with *Leutimus lepidus*,

had more bearing on the synthesis of lignin precursors. More recently the hydroxylation of *p*-coumarate to caffeate and subsequent ring cleavage by *Pseudomonas* was demonstrated by Seidman *et al.* (1969). Experiments with whole cells of *S. nigrifaciens* revealed that this organism converted *p*-coumarate into protocatechuate with the intermediate formation of caffeate, followed by degradation of the aliphatic side chain (Nambudiri *et al.*, 1969). A purified enzyme from *S. nigrifaciens* readily hydroxylated *p*-coumarate to caffeate. *o*-Hydroxylation of *p*-coumarate has also been shown to occur in higher plants (Sato, 1966; Vaughan & Butt, 1969*a*,*b*; Stafford & Baldy, 1970). A phenolase preparation from mushroom also catalysed the hydroxylation of *p*-coumarate to caffeate (Sato, 1969).

A spinach enzyme isolated by Vaughan & Butt (1967) that catalyses the hydroxylation of *p*-coumarate was found to contain both monophenol oxidase and *o*-diphenol oxidase activities, typical of a phenolase type of enzyme (Vaughan & Butt, 1969*a*,*b*). The purified *Streptomyces* enzyme also contained both *p*-coumarate hydroxylase and caffeate oxidase activities. The two activities appear to be the function of the same enzyme, as the ratio of the hydroxylase activity to the caffeate oxidase activity remained virtually the same throughout the purification procedure. The molecular weight of the *Streptomyces* enzyme, in the present studies, was estimated to be about 18000 by Sephadex gel filtration. This is the smallest tyrosinase molecule yet found in Nature and is a little over half the molecular weight of the *Neurospora* enzyme reported by Fling *et al.* (1963) as 33000 ± 2000.

The *Streptomyces* preparation hydroxylated, in addition to *p*-coumarate, *p*-hydroxyphenylpropionate, *p*-hydroxyphenylacetate, L-tyrosine and *p*-hydroxyphenyl-lactate in decreasing order of activity. In some respects this resembled in substrate specificity a monophenol oxidase isolated from sorghum (Stafford & Baldy, 1970). Although not a classical polyphenol oxidase, the sorghum enzyme catalysed the hydroxylation of *p*-coumarate. Compounds such as *p*-hydroxybenzoate, *p*-hydroxymandelate, *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde were not substrates. However, caffeate and tyrosine, which are substrates for the *Streptomyces* enzyme, were inhibitory with the sorghum preparation.

Ascorbate serves as a reductant for mono-oxygenase reactions catalysed by dopamine β-hydroxylase (Kaufman, 1966) and phenolase (Mason *et al.*, 1955). Reduced nicotinamide nucleotides, however, are obligatory participants in many other mono-oxygenase-catalysed hydroxylations (Hayaishi, 1969). 2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine was the most effective hydrogen donor for the *p*-coumarate hydroxylating enzyme from spinach, although NADH, NADPH and ascorbate also were effective (Vaughan & Butt, 1969*a*). The *p*-coumarate-hydroxylating enzyme isolated from *S. nigrifaciens* also showed a requirement for a reduced cofactor as an electron donor. In the absence of any reductant the hydroxylation proceeded only after a considerable lag period. As an electron donor, NADH, NADPH, H_4PteGlu and ascorbate were more-or-less equally effective, but at higher concentrations ascorbate was inhibitory. Such an inhibition was not observed with spinach (Vaughan & Butt, 1969*a*) and mushroom (Sato, 1969) phenolase preparations, and, in fact ascorbate was a suitable reductant for the hydroxylation of *p*-coumarate. The effectiveness of the electron donor for hydroxylation seems to depend on the nature of the substrate as well. In the present investigation, with *p*-hydroxyphenylacetate as substrate, ascorbate was the most effective hydrogen donor without exerting, at the same time, any inhibitory effect on the reaction. The reason for the differential effect of higher concentrations of ascorbate remains to be explained.

The *Streptomyces* enzyme under study contained 0.38% copper, which is similar to the copper contents reported for other phenolases (Bauchillaux *et al.*, 1963; Fling *et al.*, 1963; Kertesz & Zito, 1962; Smith & Krueger, 1962). Barron & Singer (1945) showed that phenolase is not a thiol enzyme. Pure mushroom phenolase remains fully active even in the presence of a great excess of *p*-hydroxymercuribenzoate (Kertesz & Zito, 1958). Similarly, inhibition studies on *Neurospora crassa* enzyme by Fling *et al.* (1963) also revealed that *p*-hydroxymercuribenzoate did not significantly inhibit the enzyme activity even at high concentrations. The fact that neither *p*-hydroxymercuribenzoate nor heavy-metal ions such as Hg^{2+} inhibited the *Streptomyces* enzyme suggests that free thiol groups are not involved in the reaction. In agreement with this finding thiol-reducing agents such as GSH, β-mercaptoethanol and dithiothreitol completely inhibited the enzyme activity even at a concentration of 0.1 mM. Attempts to prevent the inhibition by preincubation with Cu^{2+} were not effective. Inhibition of tyrosinase activity by GSH and cysteine was observed also by Miamoto *et al.* (1967) with a purified preparation from potato. However, unlike the *Streptomyces* enzyme, inhibition of potato phenolase was reversed by the addition of Cu^{2+}. In contrast with the *Streptomyces* enzyme, the monophenol oxidase from sorghum (Stafford & Baldy, 1970) showed an obligatory requirement for a thiol-reducing agent such as β-mercaptoethanol, GSH or dithiothreitol.

The caffeate oxidase activity of the *Streptomyces* enzyme was inhibited by some monophenols such as *p*-coumarate, *p*-hydroxyphenylacetate and tyrosine. The *o*- or *m*-hydroxy compounds had no effect. Inhibition of catecholase activity of the mammalian tyrosinase by monophenols was reported by Miamoto & Fitzpatrick (1957), and also by

Pomerantz (1963), Osaki (1963) and Kean (1964) in other phenolase systems. *p*-Coumarate is a potent inhibitor of apple phenolase (Walker, 1969). Inhibition of catecholase activity by benzoate and cinnamate has been reported (Kuttner & Wagreich, 1953). Similarly, inhibition of cresolase activity of potato tyrosinase by cinnamate, benzoate and ferulate is known (Zucker, 1966).

The *Streptomyces* enzyme seems to differ from previously described systems in that compounds such as *trans*-cinnamate and benzoate did not inhibit the monophenol oxidase or the diphenol oxidase activity. Also the *m*- and *p*-hydroxyphenols did not have any effect on the oxidation of caffeate. Pomerantz (1964) reported that the hydroxylation of tyrosine catalysed by mammalian tyrosinase exhibited an apparent substrate (tyrosine) inhibition at concentrations above 0.8mM. The rate of hydroxylation of *p*-coumarate by the *Streptomyces* enzyme, however, was not affected by high concentrations of *p*-coumarate.

Walker (1969) observed that the type of inhibition of *o*-diphenol oxidase activity of the apple tyrosinase by *p*-coumarate depended on the substrate. Catechol oxidation was competitively inhibited, whereas the inhibition of cholorogenic acid oxidation was non-competitive. Similar differences were also observed by Macrae & Duggleby (1968) in inhibition studies with apple phenolase. With the *Streptomyces* enzyme the inhibition was purely competitive when caffeate was the substrate. No attempts were made to study the pattern of inhibition with other substrates.

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