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p-Cresol methylhydroxylase

Assay and general properties

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p-Cresol methylhydroxylase from *Pseudomonas putida*, an anaerobic dehydrogenase that catalyses the oxidation of *p*-cresol to *p*-hydroxybenzyl alcohol and then to *p*-hydroxybenzaldehyde, is an enzyme of great interest in several respects. One of these is the fact that its flavoprotein and cytochrome *c* subunits may be reversibly dissociated with ease, with full regeneration of the activity and its native properties on recombining the components. Bisubstrate kinetic analysis of the unresolved enzyme gives parallel-line kinetics in double-reciprocal plots, whereas the reaction of the separated flavoprotein subunit with substrates is described by converging lines. The mechanistic implications of these behaviours are discussed. Reductive titration with dithionite results in the uptake of 3 electrons by the enzyme, with the intermediate formation of the anionic flavin radical [McIntire, Edmondson, Hopper & Singer (1981) *Biochemistry* **20**, 3068–3075]. Reductive titration with substrates resulted initially only in reduction of the cytochrome subunit, followed by formation of the anionic radical and finally the fully reduced enzyme. These observations suggest rapid intermolecular electron transfer between *p*-cresol methylhydroxylase molecules. This paper also examines the effect of pH and ionic strength on the activity and specificity of the enzyme with respect to substrates and natural, as well as artificial, electron acceptors. The absorption coefficients of the enzyme and of its subunits in various oxidation states are also presented.

p-Cresol methylhydroxylase, a flavocytochrome, catalyses the dehydrogenation and hydration of *p*-cresol and its homologues to the corresponding alcohols, which are then further dehydrogenated to the corresponding aldehydes or ketones. Thus oxidation of *p*-cresol yields *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde (Hopper, 1976, 1978). The enzyme has been isolated from *Pseudomonas putida* and a number of other bacteria (Hopper & Taylor, 1977; Keat & Hopper, 1978). The enzyme has several unusual and interesting properties. It is the only protein so far encountered to contain a covalently linked flavin with tyrosine as the substituent on C-8α of FAD (McIntire *et al.*, 1981), and to have been reversibly resolved into its flavoprotein and cytochrome subunits (McIntire & Singer, 1982). This has permitted the demonstration that the cytochrome *c* subunit regulates the catalytic activity of the flavin site in the other subunit. It also catalyses the partially asymmetric hydroxylation of 4-ethylphenol to 1-(4′-hydroxyphenyl)ethanol (McIntire *et al.*, 1984) and is the first flavocytochrome for which the rates of intramolecular electron transfer have been characterized (Bhattacharyya *et al.*, 1985).

The present paper is intended to describe the general properties of the enzyme from *Ps. putida* strain 9869, form A, as well as to provide the experimental procedures to be used, including its assay and the chemical determination of the concentration of the enzyme, in a planned series of papers dealing with this protein.

Abbreviations used: DCIP, 2,6-dichloroindophenol; PMS, phenazine methosulphate.

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Experimental

Materials

p-Cresol methylhydroxylase from *Ps. putida* N.C.I.B. 9869, form A, was purified by a method similar to those reported earlier (Hopper & Taylor, 1977; Keat & Hopper, 1978). The flavoprotein and cytochrome subunits were separated from the native flavocytochrome as previously described (McIntire & Singer, 1982). 4-Methyl-1-naphthol was purchased from ICN Pharmaceuticals. *p*-Hydroxybenzylaldehyde, *m*-cresol, L-tyrosine hydrochloride, horse heart cytochrome *c* (type VI), phenazine methosulphate (PMS), 2,6-dichloroindophenol (DCIP) and bovine liver catalase, a 23mg/ml suspension, were purchased from Sigma Chemical Co. DCIP was also obtained from General Biochemicals, and *o*-cresol from Matheson, Coleman and Bell. All other substrates and substrate analogues were from the Aldrich Chemical Co. Thin-layer silica-gel plates (HETLC–GHFL) with a fluorescent indicator were from Analtech, and silica gel 60 from E. Merck, Darmstadt, Germany. A 6.67mg/ml solution of glucose oxidase from *Aspergillus niger* was purchased from Miles Laboratories. Cytochrome *c*-551 from *Pseudomonas aerugenosa*, cytochrome *c*-554 from halophilic *Paraccocus* and cytochrome *c*-551 from *Pseudomonas stutzerii* were all gifts from Dr. T. Meyer (University of Arizona, Tucson AZ, U.S.A.) and 5-deazariboflavin was a gift from (the late) Professor P. Hemmerich (University of Konstanz, Konstanz, Germany).

Purification of substrates and analogues

Chemicals from commercial sources were purified by crystallization, distillation or sublimation. Their purity was checked by melting point, t.l.c., h.p.l.c., mass spectrometry or ^{1}H-n.m.r. spectrometry.

Synthesis of RS-1-(4-hydroxyphenyl)ethanol

A 1g portion (7.35mmol) of 4-hydroxyacetophenone was dissolved in 10ml of water and 5ml of ethanol. $NaBH_4$ (0.5g, 13.2mmol), dissolved in 5ml of water, was slowly added, with stirring. After 20min H_2 evolution ceased and the solution was acidified to pH5.0 with 25% (v/v) acetic acid, and extracted first with three 100ml portions of chloroform and then three 100ml portions of water-saturated ethyl acetate. The combined solutions were dried over Na_2SO_4, filtered, and after 18h at −20°C 0.2g of white crystals was collected by filtration. The solvent was removed from the filtrate *in vacuo*, and the solid suspended in chloroform. This mixture was heated to 50°C and enough ethanol was added to dissolve the solid. Another 0.35g of product was obtained by crystallization at −20°C. The yield of pure product was 0.55g (3.98mmol; 54%). The material migrated as a single spot on t.l.c. [silica gel with chloroform/methanol (20:1, v/v); R_F 0.31].

Enzyme assays

In routine assays activity was determined with PMS as the primary electron acceptor and DCIP as the terminal acceptor, the reduction of which was monitored spectrophotometrically at 600nm. In most kinetic studies activity was expressed as $V_{max.}$ (referred to below as V) with PMS as the varied-concentration substrate. The concentration range of PMS used varied with the phenolic substrate and is specified in the Figures and Tables.

Because of the profound effect of ionic strength on activity, buffers were prepared by adding NaCl so as to give constant ionic strength at different pH values. Thus in pH studies the ionic strength was held constant at 0.05. Sodium phosphate (16.7mM) was used at pH6.4, 7.0 and 7.6, and 50mM-Tris/HCl at pH7.6, 8.3 and 8.9. Assays in sodium phosphate and Tris/HCl buffers at pH7.6 gave identical results. At pH8.9 200μM-DCIP was used to ensure that the reaction of reduced PMS with DCIP would not be rate-limiting (McIntire, 1983). Because of the high absorbance at 600nm of the 200μM-DCIP solution, a 0.5cm-light-path cell was used. In all other assays the light path was 1cm. A saturating concentration of 0.6mM-*p*-cresol was used at all pH values.

When cytochrome *c* was substituted for DCIP its reduction was monitored at 550nm, and in experiments with O_2 as the terminal electron acceptor a Gilson Oxygraph was used.

Enzyme concentrations were calculated from the absorption coefficient of the oxidized holoenzyme at 412nm (143mM^{-1}·cm^{-1}) and of the flavoprotein subunit at 440nm (11.3mM^{-1}·cm^{-1}) (cf. the Results section). The concentration of the haem was determined as described by Bartsch (1971), by using 31.18mM^{-1}·cm^{-1} as the absorption coefficient of the reduced pyridine haemochromogen at 552nm.

Flavin analysis

A 100μl portion, of a solution of the enzyme or of the flavin subunit (45nmol/ml), was treated with 10μl of 55% (w/v) trichloroacetic acid. After 10min the mixture was centrifuged and the resulting pellet redissolved in 0.4ml of 1% (w/v) sodium dodecyl sulphate in 100mM-sodium phosphate buffer, pH7.0. A few crystals of $Na_2S_2O_4$ were added, and the test tube was flushed with argon and stoppered. The solution was left to incubate for 30min at room temperature to ensure

that the reductive cleavage of the tyrosyl-flavin ether linkage was complete (McIntire *et al.*, 1981).

Then 10 μl of 55% (w/v) trichloroacetic acid was added and the solution was heated at 100°C for 15 min to break the flavin down to the FMN/riboflavin level. The fluorescence of a sample was determined at an emission wavelength of 525 nm (excitation 445 nm). To check internal quenching, a small known amount of riboflavin standard was added and the fluorescence remeasured. The amount of flavin in the sample was calculated by comparison with a riboflavin standard.

Reductive titrations

A 1 ml portion of a 12 μM solution of the enzyme in 50 mM-sodium pyrophosphate buffer, pH 8.0, was made O_2-free by repeated evacuation and flushing with argon in a 1 cm-path-length anaerobic cuvette (Lambeth & Palmer, 1973). The flavin subunit, 30.7 nmol in 1.2 ml of 25 mM-sodium phosphate buffer, pH 7.0, was made anaerobic in the same manner. The *p*-cresol solution used as reductant contained 10 mM-glucose, 130 μg of glucose oxidase and 69 μg of catalase in 3.6 ml of 10 mM-sodium acetate buffer, pH 5.2, to ensure anaerobic conditions. The spectra were corrected for volume changes occurring on evacuation and addition of the titrant. Note that *p*-cresol is converted into *p*-hydroxybenzyl alcohol, which is also a substrate for these proteins. As a result, 1 equivalent of *p*-cresol supplies 4 electron equivalents.

Computer data analyses

Data described by non-linear functions were fit via the so-called 'weighted linear jackknife' algorithm (Fox *et al.*, 1980), as modified by Oppenheimer *et al.* (1981). The algorithm incorporated the derivative-free technique of Ralston & Jennrich (1978) and the matrix-inversion and tolerance-limitation techniques of Jennrich & Sampson (1968). All dependent variables were weighted equally.

Results

Determination of enzyme concentration

Protein concentration could not be conveniently measured by conventional methods, such as the biuret or Lowry *et al.* (1951) procedures, because of interference by the absorbance of the haem component and the low reactivity of the cytochrome subunit with the reagents. Although fluorometric analysis of free flavin provides, in principle, an unambiguous means for determining the enzyme concentration, the reductive cleavage of the covalent flavin bond (McIntire *et al.*, 1981) produces only 75–90% of the flavin expected, so that the procedure given in the Experimental section merely indicated that the flavoprotein/cytochrome ratio in the native enzyme is approx. 1:1.

Hence it was important to determine the absorption coefficients of the unresolved enzyme and of its subunits accurately, as a means of determining the absolute concentration of the enzyme. The absorption coefficient of the pure flavoprotein at 440 nm was determined as follows. A 1.1 ml portion of the protein solution (approx. 24 μM), containing 14.5 μM-EDTA, 0.49 μM-5-deazariboflavin and 100 mM-sodium phosphate buffer, pH 7.0, was made anaerobic by repeated evacuation and flushing with O_2-free argon. The absorption spectrum was recorded before and after this process in order to correct for volume changes. The solution was irradiated with two 15 W daylight fluorescent bulbs until reduction of the flavin was complete. The sample was then titrated anaerobically with a standardized solution of $K_3Fe(CN)_6$, and light-absorbance at 440 nm was monitored. The equivalence point of a plot of A_{440} versus nmol of $K_3Fe(CN)_6$ added permitted calculation of an absorption coefficient of 11.3 $mM^{-1} \cdot cm^{-1}$. The coefficients for the cytochrome subunit were determined by titration with $K_3Fe(CN)_6$ under the conditions given above, except that dithionite was used as reductant in lieu of light/EDTA/deazaflavin. The coefficients were also determined by recording the spectra of the oxidized enzyme and cytochrome subunit and then determining its concentration by the pyridine haemochromogen method (Table 1). The values for horse heart cytochrome *c* are given for comparison.

Substrate specificity

The specificity of the enzyme for the phenolic substrate was initially studied at high constant phenol concentration and varied PMS concentration. Double-reciprocal plots for some of the best substrates are shown in Fig. 1, and the values for V, K_{PMS} and V/K_{PMS} calculated from computer fit of the data to the Michaelis–Menten equation for a series of substrates are summarized in Table 2. Note that the slopes of the lines in Fig. 1 are identical, as are the corresponding V/K_{PMS} terms in Table 2.

From the sequence of reactions in the ping-pong and ordered-binding mechanisms (Scheme 1), the steady-state equation, and the definitions of V, K_{PMS} and V/K_{PMS}, are as follows:

$$v = \frac{V[S][PMS]}{K_{PMS}[S] + K_s[PMS] + [S][PMS]} \quad (1)$$

$$V = \frac{k_{+2}k_{+3}k_{+5}}{k_{+2}k_{+3} + k_{+5}(k_{+2} + k_{+3})} \quad (2)$$

Table 1. *Absorption coefficients of cytochrome c, cytochrome subunit and p-cresol methylhydroxylase*
Values in parentheses are the wavelengths. Absorption coefficients were calculated from the absorbance in 25mM-phosphate buffer, pH 7.6 and *I* 0.075, and the enzyme concentration determined by the method given. Cytochrome *c* concentration was calculated by using $\varepsilon_{552} = 31.18\,mM^{-1}\cdot cm^{-1}$ for the reduced pyridine haemochromgen (Bartsch, 1971). Abbreviations: N.D., not determined.

	Absorption coefficient ($mM^{-1}\cdot cm^{-1}$)			
		Cytochrome subunit		*p*-Cresol methylhydroxylase (haemochromogen method)
Spectral bands	Cytochrome *c* (haemochromogen method)	(haemochromogen method)	[$K_3Fe(CN)_6$ titration]	
Fully oxidized				
α	8.51 (550nm)*	6.6 (552nm)	6.39	8.04 (552nm)
β	11.6 (520nm)	10.3 (523nm)	10.0	12.2 (523nm)
γ	113 (410nm)	123 (412nm)	119	143 (412nm)
	0.897 (695nm)†	0.822 (690nm)†	N.D.	N.D.
Fully reduced				
α	29.5 (550nm)	25.6 (552nm)	24.8	27.4 (552nm)
β	17.4 (520nm)	18.1 (523nm)	17.5	20.2 (523nm)
γ	141 (414nm)	160 (417nm)	155	187 (417nm)
Reduced − oxidized				
α	21.1 (550nm)	19.0 (552nm)	18.4	19.4 (552nm)
β	5.80 (520nm)	7.75 (523nm)	7.50	7.80 (523nm)
γ	N.D.	110‡	106‡	127‡

* The value from the data of Van Gelder & Slater (1962) was 8.40.
† Computed from the ratio of absorbance at the wavelength in parenthesis to the absorbance in the 520–523nm range.
‡ Absorbance at 420nm minus that at 406nm (the maximum and minimum in the difference spectrum).

Table 2. *Steady-state parameters for various substrates of p-cresol methylhydroxylase*
All the assays were performed at 25°C in 50mM-Tris/HCl buffer, pH 7.6 and *I* 0.05. The term 'sat'. denotes that the concentration of the substrate was saturating; thus the parameters for these substrates are the true values. For the other substrates the concentrations were not saturating and the values for the parameters in these cases are apparent values. The units for V/K were calculated by using 114000 as the M_r.

Substrate	Concentration (mM)	V (μmol/min per mg)	K_{PMS} (mM)	$10^{-5} \times V/K$ ($M^{-1}\cdot min^{-1}$)
p-Cresol	1.46 (sat.)	48.6±5.6	6.76±1.17	8.30±0.58
4-Ethylphenol	1.67	13.3±0.6	1.94±0.18	7.85±0.40
2,4-Dimethylphenol	1.45 (sat.)	39.3±2.3	5.23±0.53	8.61±0.39
4-Hydroxybenzyl alcohol	0.784 (sat.)	23.6±1.5	3.26±0.37	8.44±0.40
2-Bromo-4-methylphenol	0.770 (sat.)	3.94±0.13	0.366±0.026	12.3±0.5
4-n-Propylphenol*	9.0	0.839±0.015	0.281±0.024	3.45±0.27
		0.439±0.015	0.0601±0.0069	8.37±0.75
4-Isopropylphenol	9.0	0.831±0.017	0.0264±0.0031	36.4±5.5

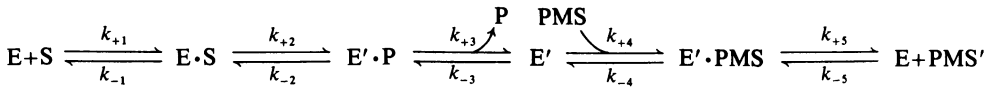
* Double-reciprocal plots for this substrate showed a downward curvature, resulting in two nearly linear regions. The values in the Table are parameters for these two regions.

$$K_{PMS} = \frac{k_{+2}k_{+3}(k_{-4}+k_{+5})}{k_{+4}[k_{+2}k_{+3}+k_{+5}(k_{+2}+k_{+3})]} \quad (3)$$

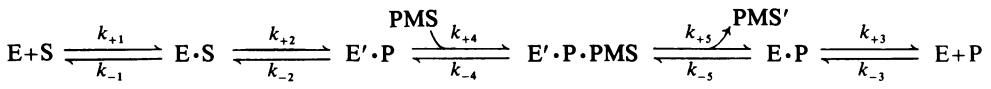
$$V/K_{PMS} = \frac{k_{+4}k_{+5}}{k_{-4}+k_{+5}} \quad (4)$$

In the derivation of these equations k_{-2}, k_{-3} and k_{-5} were all set equal to zero (see the Discussion section). Since in the ping-pong mechanism the V/K_{PMS} term contains only rate constants for the interaction of the dye with the enzyme, after the phenolic product is released, it should be the same for all substrates. On the other hand, in the ordered binding mechanism the k_{+4}, k_{-4} and k_{+5} terms represent the interactions of PMS with the reduced enzyme-product complex and thus the V/K_{PMS} ratio may be different for each substrate. The similarity of the ratios in Table 2 favours a ping-pong mechanism.

The following compounds were found to be substrates of the enzyme: 3,4-dimethylphenol, 2-



Ping-pong mechanism



Ordered-binding mechanism

Scheme 1
E′ and PMS′ are reduced forms of the enzyme and oxidant respectively.

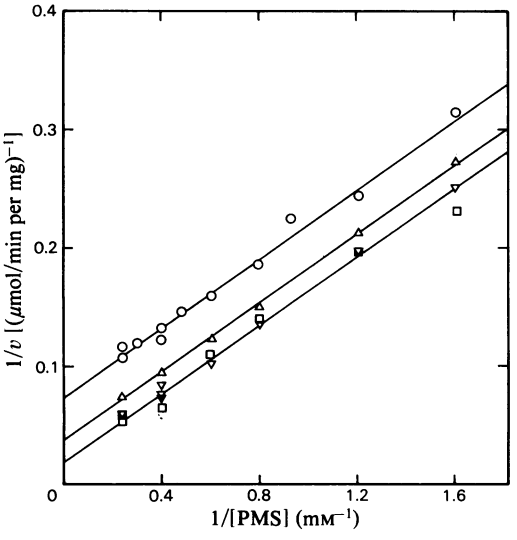


Fig. 1. *Steady-state data for various phenolic substrates of p-cresol methylhydroxylase*
All assays were carried out at 25°C in 50mM-sodium phosphate buffer, pH7.6 and *I* 0.5 (NaCl). The DCIP concentration was 100μM. The points were obtained by varying the [PMS] at constant concentrations of the phenolic substrate. The curves represent 1.47mM-*p*-cresol (□), 1.45mM-2,4-dimethylphenol (▽), 0.78mM-4-hydroxybenzyl alcohol (△) and 1.67mM-4-ethylphenol (○).

methoxy-4-methylphenol, 4-methylcatechol, 3-fluoro-4-methylphenol, 4-methyl-3-nitrophenol, *RS*-1-(4-hydroxyphenyl)ethanol, 2-bromo-4-methylphenol, 4-n-propylphenol, 4-isopropylphenol, quinol (1,4-hydroquinone) and 4-methyl-1-naphthol. Compounds that did not act as substrates included 4-methyl-2-nitrophenol, 2,4,5-trimethylphenol, 2-chloro-4,5-dimethylphenol, 2,4,6-trimethylphenol, 2,6-dibromo-4-methylphenol, L-tyrosine, 4-hydroxybenzaldehyde, *m*-cresol and *o*-cresol. It is also known that the following compounds are not processed by the enzyme (Keat & Hopper, 1978): 2,3-dimethylphenol, 2,5-dimethylphenol, 2,6-dimethylphenol, 3,5-dimethylphenol, 3-hydroxybenzyl alcohol, 2-hydroxybenzyl alcohol, 4-methoxyphenol, *RS*-1-phenylethanol, *p*-xylene, 4-hydroxyacetophenone, 1-hydroxyacetophenone, *p*-methylanisole and 4-methylbenzyl alcohol. Collectively, these observations indicate that the substrate must have an alkyl group or a 1-hydroxyalkyl group *para* to the phenolic hydroxy group. The phenolic oxygen atom cannot be blocked by alkylation, nor can it be located at the *ortho* or *meta* position. Another interesting observation is that the *p*-cresol can only be substituted at the 2- and/or 3-positions if it is to be acted upon.

From bisubstrate kinetic analysis for the flavocytochrome, as shown in Fig. 2 for 4-hydroxybenzyl alcohol and PMS, the K_s values for *p*-cresol and 4-hydroxybenzyl alcohol at pH8.0 and 30°C were calculated to be 13 and 54μM respectively. Both primary plots showed parallel lines for the unresolved flavocytochrome with either of these substrates. The isolated flavoprotein subunit (Fig. 3), however, showed marked deviation from parallel-line kinetics for both plots with all substrates tested. This rules out a ping-pong mechanism for the flavoprotein and indicates a change in kinetic mechanism on removal of the cytochrome subunit (McIntire & Singer, 1982); the implications of this are not discussed in detail in the present paper.

Reactivity with electron acceptors

The specificity of the enzyme for artificial electron acceptors is quite restricted. Molecular O_2, $K_3Fe(CN)_6$, Methylene Blue and DCIP failed to react with the enzyme at measurable rates. Recrystallized Wurster's Blue (the radical of *NNN′N′*-tetramethylphenylenediamine) reacted with the enzyme fairly rapidly, but a significant blank rate was observed, which interfered with the

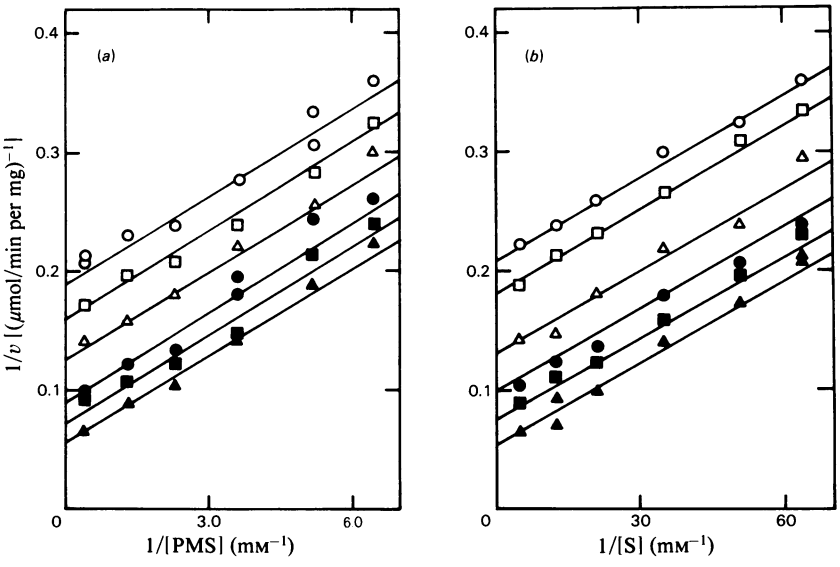


Fig. 2. *Double-reciprocal plots for the reaction of 4-hydroxybenzyl alcohol and PMS with the A form of p-cresol methylhydroxylase*

The assays were carried out in 50mM-sodium pyrophosphate buffer, pH8.0, at 30°C. (*a*) Plots of $1/v$ versus 1/[PMS] at 197 μM- (▲), 78 μM- (■), 47.1 μM- (●), 28.3 μM- (△), 19.6 μM- (□) and 15.7 μM- (○) 4-hydroxybenzyl alcohol. (*b*) Plots of $1/v$ versus 1/[S] (S = 4-hydroxybenzyl alcohol) at 2.43mM- (▲), 0.772mM- (■), 0.436mM- (●), 0.277mM- (△), 0.193mM- (□) and 0.155mM- (○) PMS.

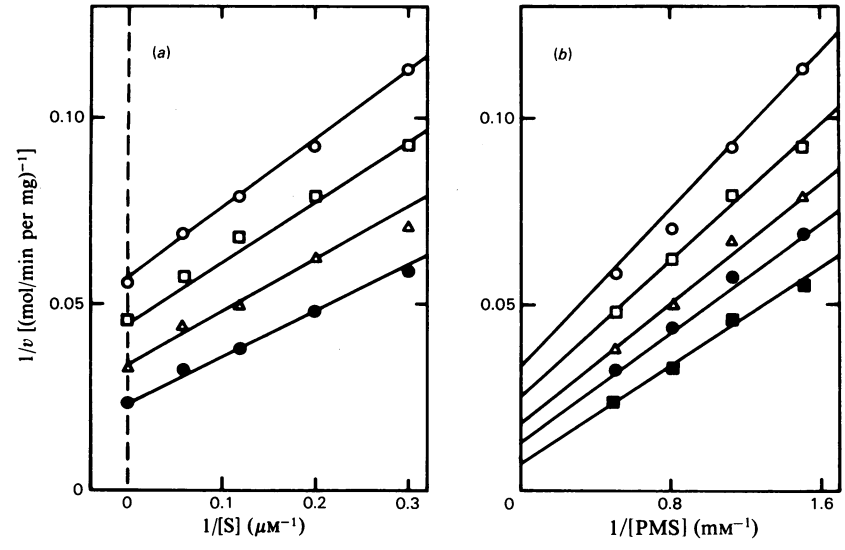


Fig. 3. *Double-reciprocal plots for the reaction of p-cresol and PMS with the flavin subunit of p-cresol methylhydroxylase*

Plots of assays performed at 25°C in 16.7mM-sodium phosphate buffer, pH7.6 and I 0.05. (*a*) Plots of $1/v$ versus 1/[S] (S = *p*-cresol) at 1.99mM- (●), 1.24mM- (△), 0.883mM- (□) and 0.663mM- (○) PMS. (*b*) Plots of $1/v$ versus 1/[PMS] at 669 μM- (■), 16.7 μM- (●), 8.37 μM- (△), 5.02 μM- (□) and 3.35 μM- (○) *p*-cresol.

assay. Horse heart cytochrome *c* was only very slowly reduced by the enzyme. As previously noted (Hopper & Taylor, 1977; Keat & Hopper, 1978), the enzyme reduces PMS rapidly, and the reaction permits the use of the conventional spectrophotometric PMS–DCIP assay.

Plots of reciprocal velocity versus reciprocal PMS concentration showed an upward curvature

(Fig. 4), and hence inhibition at high concentrations, as has been observed in the PMS–DCIP assay of other enzymes. Moreover, the blank rate in this assay (the absorbance change in the absence of enzyme), which is insignificant near neutrality, becomes appreciable as the pH is raised above 8.0 or the temperature is increased. In earlier studies (Keat & Hopper, 1978) the PMS–DCIP assay of this enzyme had been conducted at pH 9.6, a pH at which the blank rate was excessive in our hands.

In the hope of circumventing both of these complications, we tried to substitute horse heart cytochrome *c* for DCIP as the terminal electron acceptor. In the original description of the spectrophotometric PMS assay for succinate dehydrogenase, 50 μM-cytochrome *c* or 90 μM-DCIP was sufficient for saturation and they gave the same observed rate (Arrigoni & Singer, 1962; Ackrell *et al.*, 1977). For reasons we do not understand, however, in the assay of *p*-cresol methylhydroxylase the substitution of cytochrome *c* for DCIP resulted in considerably higher maximal velocity, as well as significantly higher apparent K_m for PMS (Fig. 4). Suitable control experiments showed that DCIP and cytochrome *c* were in excess not only at the pH of these experiments but also at pH values up to 8.5. Despite the 1.3–1.7-fold higher rate measured in the PMS–cytochrome *c* assay, we continued using DCIP in routine work because of its lower cost, the lower blank rates at alkaline pH values and the lesser inhibition at high concentrations (Fig. 4).

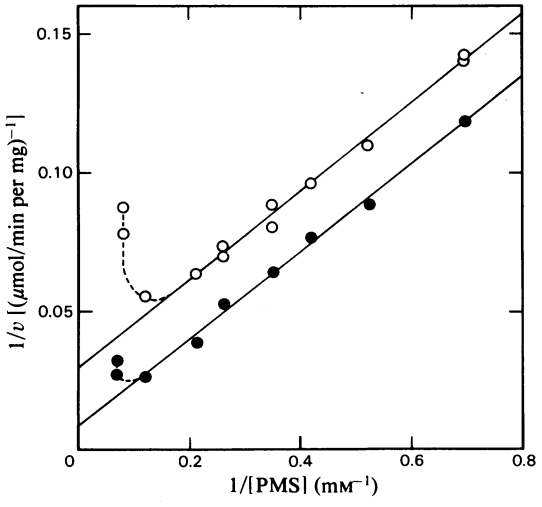


Fig. 4. *Comparison of the PMS–DCIP and the PMS–cytochrome c assays of p-cresol methylhydroxylase activity*
All assays were performed in 50mM-sodium phosphate buffer, pH 7.6 and *I* 0.5 (NaCl), at 25°C. The *p*-cresol concentration was 1.47mM, the concentration of DCIP 100 μM, and that of cytochrome *c* 56.1 μM. ○, PMS–DCIP assays; ●, PMS–cytochrome *c* assays.

Several naturally occurring electron acceptors were tested for direct reaction with the enzyme, including cytochrome *c*-551 from *Pseudomonas stutzerii*, cytochrome *c*-551 from *Pseudomonas aeruginosa* and cytochrome *c*-554 from the halophilic *Paracoccus*. The observed rates at 28 μM concentration of each of these cytochromes were 8.4, 3.4 and 0.8% of the maximum rate in the PMS–DCIP assay. At saturating concentration of the cytochromes the rates are expected to be far higher, since a study of the concentration-dependence showed that 28 μM is far below the K_m for each. As a result their use in routine assays is impractical, because of the high concentrations required and correspondingly high absorbance at the monitoring wavelength.

Preliminary experiments indicate that the copper protein azurin, from the same organism, acts as an electron acceptor for the enzyme. Since both are present in the periplasm (D. J. Hopper, unpublished work) and since azurin seems to link the enzyme to the respiratory chain of *Pseudomonas putida*, it may be a candidate for the natural acceptor of the enzyme.

Dependence of activity on pH and ionic strength

The V, K_{PMS} and V/K_{PMS} values for the enzyme were determined by measuring the steady-state velocities at various PMS concentrations at pH 6.4, 7.0, 7.6, 8.3 and 8.9, all at 25°C, an ionic strength of 0.05 and saturating *p*-cresol concentration. The activity reached a maximum at pH 8.3, fell off gradually on the low-pH side and rapidly on the high-pH side. The V/K_{PMS} ratio was constant from pH 6.4 to 8.3 and rose sharply on going to pH 8.9. It is clear from these results that pH 9 or 9.6, at which assays in previous studies had been conducted (Hopper & Taylor, 1977; Keat & Hopper, 1978), are not the optimal conditions.

On increasing the ionic strength from 0.01 to 0.1 in Tris/HCl buffer, pH 7.6, at 25°C, the maximal velocity and K_m for *p*-cresol did not change but the K_{PMS} increased substantially from 2.4 to 5.9mM. This seems to suggest that the interaction of the dye with the enzyme involves electrostatic forces. The effects of ionic strength on the isolated flavoprotein subunit was also examined. On increasing the ionic strength from 0.05 to 0.25 (50mM-Tris/HCl, pH 7.6, 25°C) the K_{PMS} declined from 5.34mM to 3.65mM, while the maximal velocity decreased modestly from 2.61 to 1.76 μmol of DCIP reduced/min per mg. Thus the effect of the ionic strength on catalytic parameters was different for the flavoprotein and the intact enzyme, possibly because in the subunit electrons flow from the reduced flavin to PMS, whereas in the intact

enzyme the reduced cytochrome transfers the electrons to PMS.

Some properties of the isolated subunits

The absorption spectrum of the cytochrome *c* subunit isolated from *p*-cresol methylhydroxylase is characteristic of *c*-type cytochromes (Table 1 and McIntire & Singer, 1982), and the spectrum of the flavoprotein subunit is typical of other flavoproteins. Fig. 5 includes spectra of the pure flavoprotein subunits in its various oxidation states. It may be noted that there is a peak at 317nm ($\varepsilon = 13.6 mM^{-1} \cdot cm^{-1}$) and a smaller one at 418nm ($\varepsilon = 2.6 mM^{-1} \cdot cm^{-1}$) in the dihydroflavin form. Most reduced flavoproteins show no well-resolved spectral features at wavelengths higher than 300nm, thioredoxin reductase being an exception, since its dihydroflavin form exhibits similar spectral features ($\lambda_{max.} = 320$ and 425nm, $\varepsilon = 9$ and $2.4 mM^{-1} \cdot cm^{-1}$ respectively; Ghisla *et al.*, 1974). The significance of these features in the reduced spectrum are considered in the Discussion section.

Fig. 5 also shows that reduction with dithionite produces a typical anionic flavoprotein radical. The concentration of the radical was not maximal, however, since during the dithionite titration slow disproportionation of the radical occurred. In order to confirm that the red (anionic) radical is produced on partial reduction, a 23.6μM solution of the enzyme in 50mM-barbital buffer, pH8.0, was titrated anaerobically with dithionite to the point of maximum absorbance at 380nm, quickly frozen, and its e.s.r. spectrum recorded. The peak-to-peak line width of 1.2mT (12G) observed is typical of anionic flavin radicals (Palmer *et al.*, 1971).

Reductive titration with substrate

Titration of the enzyme with *p*-cresol at pH 8.0 gave some unexpected results (Fig. 6). The course of the titration resembled that observed on reduction with dithionite (McIntire *et al.*, 1981). The first phase of the titration led to reduction of the haem, the second to the formation of the red (anionic) flavin radical, which was confirmed by e.s.r. (Ackrell *et al.*, 1982), and the final phase to the full reduction of the flavin. The absorbance changes produced during the second and third phases occurred relatively slowly, so that 10–20min was allowed after the addition of each increment of *p*-cresol before the spectra were recorded. Slight decreases in absorbancy at 380nm were occurring even after this interval, suggesting that disproportionation of the radical is a slow process. It may also be noted that the first phase of the titration required somewhat less (approx. 25%)

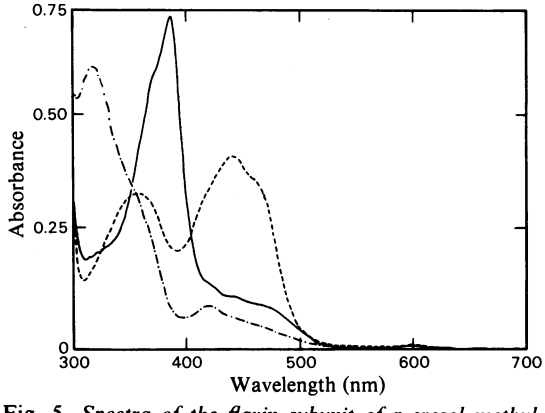


Fig. 5. *Spectra of the flavin subunit of p-cresol methylhydroxylase in various oxidation states*

The spectra were recorded for the protein in 1.0ml of 50mM-Tris/HCl, pH7.6 and *I*0.05. The solution of oxidized flavoprotein (35.5nmol) was made anaerobic and the spectrum recorded (------). The protein was titrated with standardized dithionite solution (19.7nmol of titrant added) to produce the anionic flavin radical spectrum (——). The fully reduced spectrum of the flavoprotein (–·–·–) was produced after addition of 75nmol of dithionite. Note that the reduced flavoprotein absorbance at 317nm contains a small contribution from residual dithionite. The corrected ε_{317} is $13.6 mM^{-1} \cdot cm^{-1}$.

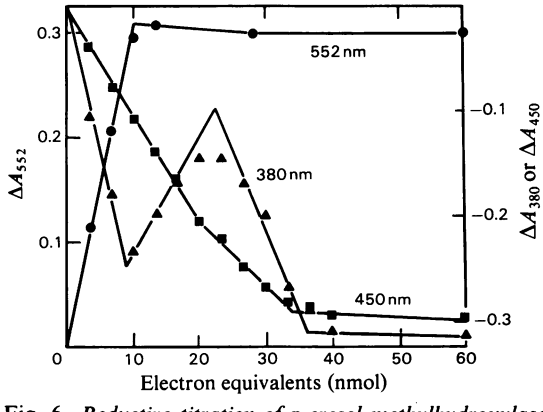


Fig. 6. *Reductive titration of p-cresol methylhydroxylase by p-cresol*

The concentration of the enzyme was 12nmol in 1.0ml of 50mM-sodium pyrophosphate buffer, pH8.0. The change in absorbance (ΔA) was calculated by subtracting the absorbance of the oxidized enzyme spectrum from the absorbance for each reduced enzyme spectrum. The plots are of the absorbance changes at 552 (●), 450 (■) and 380nm (▲) versus the nmol of electrons added. The three one-electron reduction phases, i.e. haem reduction, flavin radical formation and full reduction of flavin, are best shown by the absorbance changes at 380nm.

external reductant than did the second or third phase, indicating some reduction of the haem by the protein itself. This auto-reduction has been observed often, even after re-oxidation of the enzyme by $K_3Fe(CN)_6$.

The most noteworthy feature of these experiments is that, although the oxidation of *p*-cresol is an obligatory two-electron process, distinct one-electron reduction steps are observed. The simplest explanation is that the substrate rapidly transfers two electrons to the flavin, one of which is immediately passed on to the cytochrome subunit of the same enzyme molecule. The resulting reduced cytochrome must then be rapidly re-oxidized by the cytochrome moiety of another molecule of enzyme in order for the first one to accept the second electron from the flavin, resulting in the formation of only the ferrocytochrome form, with no indication of a flavin radical in the first phase. Rapid intermolecular electron transfer is also the most plausible explanation for the formation of the flavin radical during the second phase of the titration. Without such intermolecular electron transfer, the one-electron-reduced enzyme, in accepting two electrons from *p*-cresol, would yield dihydroflavin, rather than the radical. However, the flavin radical is formed rapidly, as indicated by an initial increase in the absorbance at 380nm on addition of each portion of substrate during the second phase. As mentioned above, the slower decrease observed at this wavelength, which required a 10–20min wait, is a result of disproportionation of the radical. The slow reduction in the final phase can also be attributed to disproportionation, since this reaction controls the concentration of enzyme containing fully oxidized flavin, the only species that can accept electrons from the substrate. Thus, besides rapid intramolecular electron transfer, the enzyme can also undergo intermolecular electron transfer.

The observations above are all the more interesting in the light of the results from the titration of the flavoprotein subunit with *p*-cresol. This protein is reduced without formation of the anionic flavin radical, i.e. only dihydroflavin is formed (results not shown). This result implies that a mechanism whereby substrate can contribute one electron at a time to the flavin (a substrate radical mechanism) cannot explain the appearance of the flavin radical in the flavocytochrome. Additionally, during stopped-flow experiments involving the flavoprotein reduction by *p*-cresol, no flavin radical was detected (McIntire, 1983), again indicating the absence of a substrate radical mechanism. These results also suggest that intermolecular electron transfer does not occur for the flavoprotein, and imply that electron transfer between flavocytochrome molecules only takes place via the haem moiety. However, as already mentioned, when the anionic flavin radical is generated in the flavoprotein subunit by dithionite reduction, this radical does slowly disproportionate, indicating the occurrence of interenzymic electron transfer. As a result, the potential of the fully reduced flavoprotein is higher than that of the radical species.

Discussion

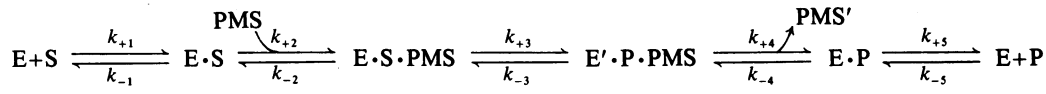
Although the steady-state kinetic data in Fig. 2 suggest that, when both subunits are present, *p*-cresol methylhydroxylase operates by a ping-pong mechanism, it is known that other mechanisms, such as rapid equilibrium and non-rapid equilibrium random systems, can also generate parallel-line kinetics under certain conditions (Dalziel, 1957; Bar-Tana & Cleland, 1974; Segel, 1975, pp. 642–643). Both random mechanisms can be ruled out for this enzyme, however, because PMS cannot accept electrons from the enzyme unless the cytochrome is reduced. This implies that the phenolic substrates must bind to the protein before the electron acceptor. This is analogous to the reoxidation of D-lactate dehydrogenase by $K_3Fe(CN)_6$ (Morpeth & Massey, 1982).

Parallel lines may also be observed in double-reciprocal plots in cases where the classical ordered-binding mechanism operates, provided that the K_i value is very small in the steady-state equation describing ordered binding mechanisms (Segel, 1975, pp. 560–565):

$$v = \frac{V[\mathrm{PMS}][\mathrm{S}]}{K_i K_{\mathrm{PMS}} + K_{\mathrm{PMS}}[\mathrm{S}] + K_s[\mathrm{PMS}] + [\mathrm{S}][\mathrm{PMS}]} \quad (5)$$

This mechanism may also be eliminated, however, because the constancy of the V/K_{PMS} ratios for several substrates (Table 2) is not expected for this mechanism.

An alternative ordered binding mechanism is shown in Scheme 1. The term k_{-5} here is set equal to zero, because [PMS′], the concentration of the reduced dye, is zero by virtue of its rapid re-oxidation by DCIP, and k_{-3} is zero, being a function of the product concentration, which is zero at $t = 0$. Further, the term k_{-2} may also be considered as zero, because reversal of the ES→E′P step seems thermodynamically unfavourable, as judged by the fact that a limiting amount of substrate is completely oxidized by an excess of enzyme (cf. Fig. 6 and the text). With these simplifications, the modified ordered mechanism described by eqns. (1)–(4), which also describe the ping-pong mechanism (Scheme 1), cannot be completely ruled out. The shortcoming of this interpretation is the constancy of the V/K_{PMS} ratio referred to above (see Table 2). This would not be



Scheme 2
PMS′ is the reduced form of the oxidant.

expected, since each substrate would yield different E′P species with which the oxidant reacts, unless the reaction site of PMS in the intact enzyme is far enough removed from the substrate-binding site that the nature of the product bound does not materially influence the reactivity with PMS. On balance, then, the ping-pong mechanism is the most likely one to be operative, although the modified ordered-binding mechanism remains a possibility.

As is apparent from Fig. 3, the steady-state kinetics of the isolated flavoprotein subunit differ materially from those of the unresolved enzyme. Therefore the rate equation for the flavoprotein subunit is also different. Stopped-flow data (McIntire, 1983) indicate that the reduction of the flavoprotein by the substrate is reversible and, as a result, k_{-2} in Scheme 1 is not zero. The terms k_{-3} and k_{-5} can be considered zero because the product concentration is zero at $t=0$ and [PMS′]$\sim$0 because of its fast re-oxidation by DCIP. These limitations taken together result in a steady-state velocity described by eqn. (5).

An alternative ordered-binding mechanism is given in Scheme 2. The reason for considering this mechanism is that stopped-flow experiments on the flavoprotein have shown that the reduction of the flavin by *p*-cresol is slow (McIntire, 1983). Consequently, PMS may be bound to the enzyme before or while the substrate is oxidized. For this mechanism the expression given by eqn. (5) also applies. For reasons given above, k_{-3} and k_{-4} are again set equal to zero. The data presented in this paper do not permit a clear distinction between the mechanisms given in Schemes 1 and 2. Steady-state isotope-effect studies could be used to distinguish between these two mechanisms.

Besides the differences in kinetic mechanism between the isolated flavoprotein and the unresolved enzyme, implied by Figs. 2 and 3, the maximal rates of oxidation of *p*-cresol also differ by a factor of 50-fold between the two preparations (McIntire & Singer, 1982). Since reduction of the flavin by the substrates is slower in the case of the isolated flavoprotein, it seems that combination with cytochrome *c* subunit modulates the reactivity of the flavin site (McIntire & Singer, 1982). Significantly, conditions have been found for the full reconstitution of the enzyme from its subunits, with return of all of the original catalytic activity and kinetic mechanism (Koerber *et al.*, 1985).

There remain two sets of interesting observations yet to be discussed. The first one concerns the absorption spectra of the flavoprotein in various oxidation states. The spectrum of the oxidized flavoprotein subunit shows some resolution of the 440nm peak into its constituent absorption bands (Fig. 5), indicating that the flavin site is somewhat non-polar (Vissar & Müller, 1979). The spectrum of the fully reduced flavoprotein is more unusual, in that its absorption maxima at 418 and 317nm correlate best with the spectrum of flavin frozen in ethanol glass (Ghisla *et al.*, 1974). The wavelength of maximal absorbance of the enzyme are shifted relative to the free flavin in the ethanol glass, by 21nm (317nm band) and 14nm (418nm band), suggesting that the protein-bound chromophore is less bent [$\pi\rightarrow\pi^*$ transitions are more allowed than in the fully folded dihydroflavin (Ghisla *et al.*, 1974)] and is more constrained than is customarily seen for reduced flavins bound to other flavoproteins.

The one-electron-reduced flavocytochrome also shows some unusual spectral changes on progressive reduction by substrate. At the start of the experiment the cytochrome is already reduced and the two electrons from substrate must enter the flavin ring system. Instead of the appearance of dihydroflavin being observed, the spectrum indicates that the anionic flavin radical is formed after addition of increments of substrate. This implies relatively fast transfer of one electron from the dihydroflavin, initially formed, to another flavocytochrome molecule, which is in the flavoquinone state. This is followed by a slow disproportionation of the radical to produce fully oxidized and fully reduced protein-bound flavin. The reaction is slow enough to be conveniently followed spectrophotometrically. A possible scheme is given below:

$$\mathrm{Cyt._{red.}Fl._{ox.}} + \mathrm{S} \xrightarrow[\text{Rapid}]{\nearrow \mathrm{P}} \mathrm{Cyt._{red.}Fl._{red.}}$$

$$\mathrm{Cyt._{red.}Fl._{ox.}} \underset{\text{Fast}}{\rightleftharpoons} 2\,\mathrm{Cyt._{red.}Fl.^{\cdot -}} \overset{\text{Slow}}{\rightleftharpoons} \mathrm{Cyt._{red.}Fl._{red.}} + \mathrm{Cyt._{red.}Fl._{ox.}}$$

where Cyt. and Fl. represent cytochrome and flavin respectively. Although this scheme describes the events actually observed (with the assumption that the initial species formed is the fully reduced enzyme, as has been verified with the flavoprotein subunit), it seems to violate the principle of microscopic reversibility. It is not inconceivable, however, that one of the flavocytochrome species formed on slow disproportionation of the radical differs in some respects from that found during initial reduction, and hence would have different oxidation–reduction potentials.

Unfortunately, the interesting oxidation–reduction chemistry described in the present paper cannot be discussed in terms of the midpoint potentials of the flavin and haem. Although the potentials of the haem component in the flavocytochrome and the isolated cytochrome subunit have been measured (250mV and 180mV respectively; Hopper, 1983), the appropriate conditions for measurement of this parameter for the flavin moiety in either the flavocytochrome or the free flavin subunit have not yet been found.

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