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Formation and properties of flavoprotein–cytochrome hybrids by recombination of subunits from different species

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p-Cresol methylhydroxylases from four different pseudomonads differ in their isoelectric points and, to a lesser extent, in M_r values and substrate specificity. The enzymes from three species were isolated in homogeneous form, then resolved into their flavoprotein and cytochrome subunits, and the subunits were recombined to yield the nine possible hybrids (i.e. three intraspecies and six interspecies). The resulting flavocytochromes showed extensive similarities in steady-state kinetic parameters and in the dissociation constants of their subunits. Evidence is also presented that a fourth type of *p*-cresol methylhydroxylase, from *Pseudomonas putida* (N.C.I.B. 9869, form 'B'), the subunits of which cannot be isolated by the isoelectric focusing technique used to separate the subunits of the other flavocytochromes, nevertheless dissociates slowly at high dilution. The dissociation is reflected by a decline of catalytic activity with time. This process for the 'B' enzyme is prevented by the presence of substrate or an excess of a cytochrome subunit isolated from another enzyme species. Incubation of the dissociated subunits with *p*-cresol brings about extensive, albeit incomplete, re-association and regeneration of activity.

INTRODUCTION

The flavocytochrome *p*-cresol methylhydroxylase (PCMH) catalyses the dehydrogenation and subsequent hydration of *p*-cresol and various homologues to the corresponding alcohols and further dehydrogenation to the corresponding aldehydes and ketones (Hopper & Taylor, 1977; Keat & Hopper, 1978; McIntire *et al.*, 1984; Koerber *et al.*, 1985). To date six forms of the enzyme have been isolated from five bacterial sources: *Pseudomonas putida* (N.C.I.B. 9866 and 9869, the latter of which produces the so-called 'A' and 'B' forms under growth conditions of 3,5-xylenol or *p*-cresol as the carbon source respectively); *Pseudomonas alcaligenes* (N.C.I.B. 9867), *Pseudomonas testosteroni* (N.C.I.B. 8955) and an *Alcaligenes* species. A preliminary comparison of the protein chemistry of these enzymes has been given (McIntire *et al.*, 1984). The enzyme isolated from *Ps. putida* (N.C.I.B. 9869), which contains two unequal subunits, has been the most extensively studied PCMH species with respect to quaternary structure and function. One subunit (M_r 48000) contains a covalently bound flavin prosthetic group (an 8-α-*O*-tyrosyl-FAD) and the other subunit (M_r 8600) is a *c*-type cytochrome. Previous studies on this enzyme have detailed the identification and synthesis of the unique tyrosyl–flavin linkage in the flavoprotein (McIntire *et al.*, 1981), the catalytic and molecular properties of the enzyme (McIntire *et al.*, 1985), the fast reaction kinetics as studied by laser flash photolysis (Bhattacharyya *et al.*, 1985), the chirality of the alcohol formed in the first dehydrogenation–dehydration step (McIntire *et al.*, 1985) and the crystallization and preliminary X-ray-crystallographic structure of the enzyme (Shamala *et al.*, 1985).

McIntire & Singer (1982) reported the resolution of the flavoprotein and cytochrome subunits of PCMH 9869A by isoelectric focusing under non-denaturing conditions and reconstitution of the active enzyme from the separated subunits. A detailed account of the flavoprotein–cytochrome interactions, including conditions for the complete reconstitution of the flavocytochrome, has been published (Koerber *et al.*, 1985). The present study expands the methodology of the isolation of the subunits and of the reconstitution of the flavocytochrome to the PCMH enzymes obtained from *Ps. putida* (N.C.I.B. 9866) and *Ps. alcaligenes* (N.C.I.B. 9867), and demonstrates that all nine possible flavoprotein–cytochrome hybrids are catalytically active and show a remarkable similarity with respect to steady-state kinetic parameters and binding forces between the flavoprotein and cytochrome subunits. The present study also considers PCMH 9869B, the individual subunits of which cannot be separated and isolated by conventional isoelectric focusing. Evidence is presented, however, that this flavocytochrome also dissociates and can be reconstituted, but that the reconstitution is not fully reversible.

EXPERIMENTAL

Materials

Folin–Ciocalteu phenol reagent and Na_2CO_3 were from the Sigma Chemical Co. All other materials were as indicated in previous work (McIntire *et al.*, 1985).

Abbreviations used were as follows. The four native *p*-cresol methylhydroxylases discussed are designated by suffixing the N.C.I.B. registry number to the enzyme abbreviation; e.g., PCMH 9866 represents the enzyme isolated from *Pseudomonas putida* (N.C.I.B. 9866). The two forms of PCMH isolated from *Ps. putida* (N.C.I.B. 9869) when that organism is grown on 3,5-xylenol or *p*-cresol are designated PCMH 9869A and PCMH 9869B respectively. The nine hybrid enzyme species resulting from the recombination of PCMH 9866, 9867 and 9869A are designated by the constituent flavoprotein (F) and cytochrome (C) components, each subscripted with the subunit source; e.g., $F_{66}C_{69}$ contains the flavoprotein of PCMH 9866 and the cytochrome of PCMH 9869A.

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Table 1. Flavoprotein–cytochrome dissociation constants

The K_D values for the recombined hybrids of PCMH were determined by holding [flavoprotein] constant and varying [cytochrome]. The incubations were in 10 mM-potassium phosphate buffer, pH 7.6 and I 0.07 (adjusted with KCl), at 4.0 °C. Specific activity was determined by the standard phenazine methosulphate/2,6-dichloroindophenol assay method. K_D values were determined by a computer fit to the binding isotherm (described in Koerber *et al.*, 1985). The estimated error was ±5%, for means of three determinations.

		K_D (nM)		
Flavin constituent	Cytochrome constituent...	C_{66}	C_{67}	C_{69}
F_{66}		63.0	101.0	28.1
F_{67}		8.7	8.4	7.5
F_{69}		24.3	101.0	7.4

Growth of organisms

Ps. putida (N.C.I.B. 9866) was grown by the method of Hopper & Taylor (1977), with 2,4-xylenol as the carbon source. *Ps. alcaligenes* (N.C.I.B. 9867) was grown by initial inoculation into a medium containing 0.1% (w/v) sodium succinate and 0.03% (w/v) *p*-cresol. This cell growth was subcultured in three stages into medium containing 0.03% *p*-cresol as the sole carbon source. The final inoculation (six 12-litre New Brunswick Scientific fermentors) at 30 °C also contained 0.03% *p*-cresol. The concentration of *p*-cresol was monitored spectrophotometrically by mixing 1 vol. of incubation mixture with 1 vol. of phenol reagent and 2 vol. of 20% (w/v) Na_2CO_3, allowing the colour to develop, and comparing the absorbance at 600 nm with a standard curve (Bray & Thorpe, 1954). The concentration of *p*-cresol was maintained above 0.02% for the 72 h incubation. *Ps. putida* (N.C.I.B. 9869) was grown by the method of Keat & Hopper (1978), with either 3,5-xylenol (form A) or *p*-cresol (form B) as the sole carbon source. Cells were harvested by continuous-flow centrifugation and stored at −20 °C.

Enzyme purification and subunit isolation

PCMH was isolated from the organisms *Ps. putida* (N.C.I.B. 9866 and 9869, forms A and B) and *Ps. alcaligenes* (N.C.I.B. 9867) by the method of Koerber *et al.* (1985). Interestingly, all four PCMH enzymes (9866, 9867, 9869A and 9869B) may be isolated by the same procedure in comparable yields. Resolution of the flavoprotein and cytochrome subunits of PCMH 9867 and PCMH 9869A was performed by a modification of the method of Koerber *et al.* (1985), with Sephadex G-200 (superfine grade) as the support matrix and an amphoteric mixture of small-molecule buffer salts to establish the isoelectric-focusing gradient. In this modification the separated subunit solutions were freed of the amphoteric buffer salts by repeated washing with 10 mM-potassium phosphate buffer, pH 7.6, and concentration in a centrifugal microconcentrator (Amicon Corp.). It was found that the flavoprotein subunit was easily concentrated by using a YM-30 (hydrophobic) membrane; however, full retention of the cytochrome subunit required a YM-10 membrane. Although satisfactory for the other forms of PCMH, in the case of PCMH 9866 this isoelectric-focusing method resulted in incomplete separation of the subunits. Consequently, for PCMH 9866 the method of McIntire & Singer (1982) was employed, which uses ampholytes (Pharmacia) to establish the isoelectric-focusing gradient in a Sephadex G-200 matrix to achieve total separation of the subunits. The subunits were freed of ampholytes by repeated washing and concentration as described above for PCMH 9867 and PCMH 9869.

Other methods

Enzyme activity was determined with the modified phenazine methosulphate/2,6-dichloroindophenol assay method of McIntire *et al.* (1985) with 50 mM-Tris/HCl buffer, pH 7.6 and I 0.05, at 25 °C. Dissociation constants for the various hybrid flavocytochrome species were determined by titration of the flavoprotein with the cytochrome subunit (Koerber *et al.*, 1985). Dissociation constants for the flavoprotein and cytochrome subunits of PCMH 9869B, determined under equimolar concentration conditions of flavoprotein and cytochrome, were fit to a simple bimolecular dissociation equation:

$$K_D = \frac{[Ft-FC]\,[Ct-FC]}{[FC]} = \frac{([FC_0]-[FC_{inf}])^2}{[FC_{inf}]} \quad (1)$$

where [Ft] (= [Ct]) is the total concentration of flavoprotein (or cytochrome) added to the incubation mixture, [FC] is the equilibrium concentration of active flavocytochrome, and $[FC_0]$ and $[FC_{inf}]$ respectively are proportional to the initial and final specific activities measured in the standard enzyme assay.

RESULTS

Formation of flavoprotein–cytochrome hybrids

Previous studies with native and reconstituted PCMH 9869A (Koerber *et al.*, 1985) showed that elevated ionic strength and low temperature tend to favour association of the subunits. Under appropriate incubation conditions, 100% of the activity of the reconstituted enzyme can be regained relative to the native enzyme. In dilute solutions it is possible to titrate the flavoprotein subunit with the cytochrome subunit and thus establish the dissociation constant. The flavoprotein–cytochrome dissociation constants for the various hybrids are given in Table 1. This Table indicates that all of the dissociation constants lie in the nanomolar range, spanning the values 7.4 nM (for the $F_{69}C_{69}$ hybrid) to 101 nM (for both $F_{66}C_{67}$ and

Table 2. Steady-state kinetic constants for reconstituted hybrids of PCMH

The steady-state kinetic parameters were measured by extrapolation to infinite concentration of phenazine methosulphate and either *p*-cresol or 2-bromo-4-methylphenol (2-BMP) in the standard phenazine methosulphate/2,6-dichloroindophenol assay method. Units were as follows $V_{max.}$ (μmol/min per mg of enzyme, based upon flavoprotein concentration in the hybrid flavocytochrome); K_m (S) (i.e. K_m for *p*-cresol or 2-bromo-4-methylphenol) (μM); K_m (PMS) (i.e. K_m for phenazine methosulphate) (mM). The estimated errors were ±3% for $V_{max.}$ and K_m (S) and ±6% for K_m (PMS), for means of three determinations.

	Cytochrome component...	C_{66}		C_{67}		C_{69}	
Flavin component	Kinetic constant	*p*-Cresol	2-BMP	*p*-Cresol	2-BMP	*p*-Cresol	2-BMP
F_{66}	$V_{max.}$	52.9	4.72	47.8	3.62	45.7	4.70
	K_m (S)	26.0	3.47	27.0	1.89	25.3	3.22
	K_m (PMS)	5.6	0.50	4.7	0.39	3.9	0.33
F_{67}	$V_{max.}$	59.1	3.67	51.6	4.64	51.1	3.21
	K_m (S)	22.5	2.59	18.4	2.59	17.2	1.77
	K_m (PMS)	7.5	0.35	6.4	0.45	5.0	0.35
F_{69}	$V_{max.}$	64.9	4.68	51.2	3.85	67.9	4.44
	K_m (S)	26.0	1.97	14.5	2.41	18.7	2.88
	K_m (PMS)	7.5	0.43	4.6	0.31	6.4	0.31

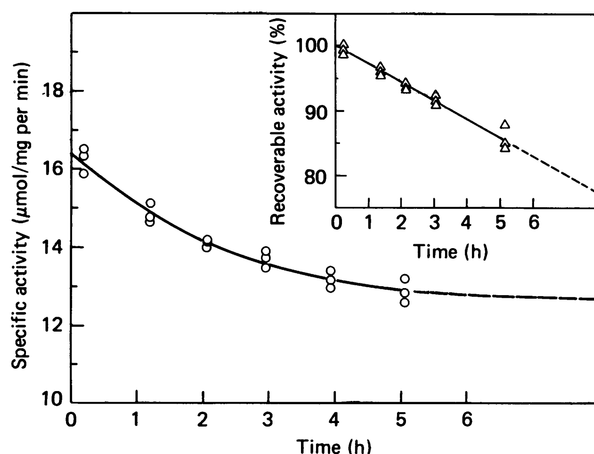

Fig. 1. Decrease of specific activity of PCMH 9869B with time after dilution

A solution of PCMH 9869B (17.4 μM) was diluted to 52.0 nM in 10 mM-potassium phosphate buffer, pH 7.6 and *I* 0.030, at 25 °C, and the specific activity (μmol/min per mg) was measured with time by the phenazine methosulphate/2,6-dichloroindophenol assay method. Inset: percentage of activity recovered (relative to the initial activity) in portions removed at different times, made 1 mM in *p*-cresol, incubated an additional 3 h, and re-assayed.

$F_{69}C_{67}$). Although all of the flavoprotein and cytochrome subunits demonstrated very high affinity for each other, as shown by the low K_D values, minor variability was apparent and presumably reflects subtle differences in protein–protein interactions. Thus the dissociation constants of hybrids containing either F_{66} or F_{69} show significant differences on recombination with the three cytochromes; however, hybrids of F_{67} show K_D values comparable with those of any of the cytochrome subunits. Such conclusions are not as clear for the cytochrome subunits; however, it appears that C_{69} binds more tightly to any given flavoprotein than does either C_{66} or C_{67}. Interestingly, C_{67} binds with more than an order of magnitude more affinity to F_{67} (the naturally occurring flavoprotein component of the flavocytochrome) than to either F_{66} or F_{69}.

Kinetic constants of the hybrids

The steady-state kinetic constants characterizing the hydroxylation of *p*-cresol and 2-bromo-4-methylphenol by the nine PCMH hybrid species are presented in Table 2. It has been shown that PCMH 9868A operates via a ping-pong kinetic mechanism with the substrates *p*-cresol and phenazine methosulphate (McIntire *et al.*, 1985). This is apparently the case for 2-bromo-4-methylphenol and phenazine methosulphate with each of the nine flavocytochrome hybrids as judged by the observation of parallel reciprocal plots (not shown). The most striking fact about the data in Table 2 is the similarity of the $V_{max.}$, K_m (S) and K_m (PMS) values of each of the nine hybrid flavocytochrome species with respect to either *p*-cresol or 2-bromo-4-methylphenol. For *p*-cresol, $V_{max.}$ (obtained by extrapolation to infinite concentration of both *p*-cresol and phenazine methosulphate) lies in the range 45.7 μmol/min per mg for the $F_{66}C_{69}$ hybrid to 67.9 μmol/min per mg for the $F_{69}C_{69}$ hybrid. For 2-bromo-4-methylphenol, the relative change in $V_{max.}$ is even less, spanning the range 3.21 μmol/min per mg for the $F_{67}C_{69}$ hybrid to 4.72 μmol/min per mg for the $F_{66}C_{66}$ hybrid.

Evidence for the dissociation of PCMH 9869B

In previous studies (McIntire *et al.*, 1984; S. C. Koerber, W. S. McIntire & T. P. Singer, unpublished work) we have noted that, under the conditions of isoelectric focusing used to separate other forms of PCMH, PCMH 9869B does not yield the individual subunits as separate bands. With the oxidized flavocytochrome, two ill-defined bands (pI approx. 5.7 and 5.8) were observed after extensive focusing. For the reduced species, a single broad band centred around a pH value of 5.6 was observed. The similarities between PCMH 9869A and PCMH 9869B in

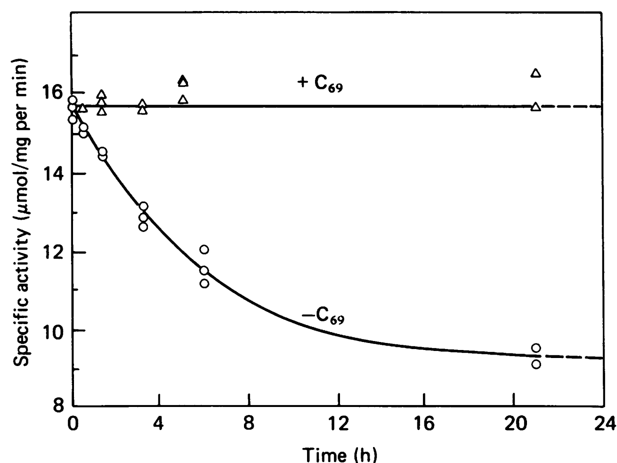

Fig. 2. Effect of exogenous cytochrome subunit on the dissociation of PCMH 9869B

PCMH 9869B (17.4 μM) was diluted to 31.6 nM with 10 mM-potassium phosphate buffer, pH 7.6 and I 0.030, at 25 °C, in the presence (upper curve) or in the absence (lower curve) of the cytochrome subunit of PCMH 9869A (819 nM), and the specific activity was determined at different times.

other respects (reactivity with *p*-cresol, M_r, prosthetic-group composition and apparent pI of the undissociated flavocytochromes) suggested the possibility that PCMH 9869B might actually dissociate, but that the pI values of the subunits are so similar that isoelectric focusing cannot resolve them.

To test this possibility, the kinetics of the loss of specific activity of PCMH 9869B upon dilution were followed by the method of Koerber *et al.* (1985). As shown in Fig. 1 (main curve), upon dilution to nanomolar concentrations PCMH 9869B loses activity in a manner consistent with a first-order dissociation process. Extrapolating the curve in Fig. 1 to zero and infinite time allowed a determination of the apparent dissociation constant. Under the experimental conditions, 10 mM-potassium phosphate buffer, pH 7.6 and I 0.03, at 25.0 °C, the K_D is approx. 10 nM. If *p*-cresol was included in the incubation mixture at zero time, no decrease in specific activity was observed with time (results not shown). This is strikingly similar to our previous observations on PCMH 9869A, showing that the dissociation of the flavocytochrome is markedly decreased if the subunits were reduced by substrate or dithionite, or if a substrate analogue incapable of undergoing oxidation is present (Koerber *et al.*, 1985).

Portions of the reaction mixture removed at intervals were incubated for 3 h with 1 mM-*p*-cresol at 25 °C, re-assayed, and a significant recovery of the activity was noted. The 3 h additional incubation time allowed the system to reach a new equilibrium. The re-association, as judged by recovery of catalytic activity, was incomplete, however; the longer the period of dissociation before the addition of *p*-cresol, the less complete was the return of activity. This is shown in the inset to Fig. 1, which gives the fraction of the initial activity recovered by the addition of *p*-cresol to the PCMH 9869B incubation mixture, as a function of the time of incubation before the addition of substrate.

If the flavoprotein and cytochrome of PCMH 9869B were undergoing dissociation during the course of the dilution procedure, it followed that the addition of an excess of cytochrome subunit isolated from another type of PCMH might force the equilibrium to the heterodimeric flavocytochrome if the exogenous cytochrome subunit were able to bind the PCMH 9869B flavoprotein. Fig. 2 illustrates such an experiment. The lower curve is the time course of the loss of activity of PCMH 9869B upon dilution, and the upper curve is the same experiment with 819 nM-C_{69} present. Even after 24 h incubation no appreciable loss of activity was observed in the presence of excess cytochrome subunit.

DISCUSSION

p-Cresol methylhydroxylases from different bacterial sources are distinguished by their isoelectric points and (to a lesser degree) by differences in M_r value and specific activity with various substrates. Further, the PCMH 9869A gene is plasmid-encoded but is not induced by 3,5-xylenol (Hopper & Kemp, 1980). PCMH 9869B, on the other hand, is genomic, induced by *p*-cresol, and functions in the protocatechuate pathway (Stanisich & Richards, 1975; Dagley, 1982). Thus it seems clear that the different PCMH enzymes are distinct proteins, probably differing in primary structure rather than being conformational isomers. It is of considerable interest, therefore, that each of the three flavoprotein subunits isolated in the present study recombines readily with any of the three cytochrome subunits, yielding fully active enzymes with comparable kinetic parameters and K_D values. Conversely, each of the cytochromes combines readily with any of the three flavoproteins isolated.

It should be noted, however, that horse heart cytochrome *c* cannot replace the *Pseudomonas* cytochromes in this regard, since the isolated flavoproteins do not reduce mammalian cytochrome *c* upon incubation of the two proteins with *p*-cresol, as would be expected if a heterodimer were formed (W. S. McIntire, C. W. Bohmont & T. P. Singer, unpublished work). In contrast, the flavocytochrome PCMH 9869A rapidly reduces horse heart cytochrome *c* in the presence of *p*-cresol (W. S. McIntire, C. W. Bohmont & T. P. Singer, unpublished work).

It is known that the flavocytochrome PCMH 9869A shows about a 50-fold increase in turnover number in the *p*-cresol/phenazine methosulphate/2,6-dichloroindophenol assay method over the purified flavoprotein subunit (McIntire, 1983), and it has been concluded that combination with the cytochrome modulates the catalytic activity of the flavoprotein. It is nevertheless likely that the relative rates of oxidation of different substrates by a given PCMH and/or their K_m values are determined by the structure of the substrate-binding site, which must be on the flavoprotein subunit (McIntire *et al.*, 1985). At the outset of this investigation it was hoped that comparison of the steady-state kinetic parameters of the different heterodimers might provide evidence for this. Despite the large differences in the rates of oxidation of the two substrates compared in Table 2, the ratios of $V_{max.}$ values for *p*-cresol and 2-bromo-4-methylphenol are not different enough in the nine hybrids to provide evidence for this. Limited studies with three other substrates [4-ethylphenol, 4-hydroxybenzyl alcohol and 1-(4′-hydroxyphenol)ethanol; results not shown] also failed to give sufficiently large differences in the ratios of $V_{max.}$ or K_m

values to prove this hypothesis. Resolution of the role of the substrate-binding site in the turnover of variously substituted phenols and the modulation of activity conferred by cytochrome binding may have to await the high-resolution X-ray-crystallographic structures of the flavoprotein and flavocytochrome species. Our ongoing collaborative efforts to this end with Dr. Scott Mathews (Shamala *et al.*, 1985) should offer much insight to the enzymology of flavocytochromes.

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