Resolution of the Flavocytochrome *p*-Cresol Methylhydroxylase into Subunits and Reconstitution of the Enzyme†

Steven C. Koerber, William McIntire, Craig Bohmont, and Thomas P. Singer*

Departments of Biochemistry and Biophysics and of Pharmaceutical Chemistry, University of California, San Francisco, California 94143, and Molecular Biology Division, Veterans Administration Medical Center, San Francisco, California 94121

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ABSTRACT: An improved procedure is described for the isolation of the flavocytochrome *p*-cresol methylhydroxylase (PCMH) from *Pseudomonas putida* as well as methods for the separation of its subunits in native form and their recombination to reconstitute the original flavocytochrome. Under appropriate conditions, the reconstitution is stoichiometric and results in complete recovery of the catalytic activity of the flavocytochrome. The separated flavoprotein subunit shows only 2% of the catalytic activity of the original enzyme on *p*-cresol and is characterized by converging lines in bisubstrate kinetic analysis, while the intact and reconstituted enzymes show parallel line kinetics in steady-state experiments. van't Hoff plots of the dependence of the dissociation constant of the subunits of PCMH on temperature show a break near 15 °C. Above this temperature, K_D is characterized by a positive ΔH value of 12.6 kcal mol^{-1}; below 15 °C, the dissociation is essentially temperature independent. The subunit dissociation is stongly dependent on ionic strength in the oxidized form of PCMH but not in the reduced form of the enzyme. Reduction also lowers the K_D significantly, while substrates and nonoxidizable competitive inhibitors lower the dissociation constant even further, suggesting a conformation change. Combination of the subunits to form PCMH entails a small but measurable change in the absorption spectra of the component proteins.

p-Cresol methylhydroxylase (PCMH),[1] a flavocytochrome which has been isolated from various pseudomonads and other bacteria (Hopper & Taylor, 1977; Keat & Hopper, 1978), catalyzes the dehydrogenation and hydration of *p*-cresol and of its homologues to the corresponding alcohols and their further dehydrogenation to the corresponding aldehydes or ketones. Thus, *p*-cresol is oxidized without the participation of exogenous O_2 to *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde (Hopper, 1976, 1978). The enzyme contains four subunits, two of approximately 49 000 daltons containing a covalently bound flavin (8α-*O*-tyrosyl-FAD) and two of a *c*-type cytochrome (~8600 daltons; F. S. Mathews, W. McIntire, and T. P. Singer, unpublished observations). Previous papers in this series have dealt with the identification and synthesis of its unusual flavin component (McIntire et al., 1981), the catalytic and molecular properties of the enzyme (McIntire et al., 1985), the reduction kinetics as studied by laser flash photolysis (Bhattacharyya et al., 1985), the chirality of the alcohol formed in the first dehydrogenation–hydration step of *p*-ethylphenol (McIntire et al., 1985), and the crystallization and preliminary X-ray study of the enzyme (Shamala et al., 1985). We have also reported briefly that the flavocytochrome is readily resolved into its native subunits, which are nearly devoid of catalytic activity but which may be recombined with regeneration of the original activity and other properties (McIntire & Singer, 1982), by isoelectric focusing. The present paper is a detailed account of the reversible resolution process, which is unique among flavocytochromes. It includes a study of the environmental factors which govern the dissociation of the subunits, discusses the conclusions which these data suggest as to the forces binding the subunits in the native enzyme, and presents for the first time evidence that reconstitution is quantitative and results in the same kinetic parameters as are shown by the intact enzyme.

Experimental Procedures

Materials. Pancreatic deoxyribonuclease I was from Sigma Chemical Co. (type III); Sephadex G-200 Superfine (Pharmacia) for isoelectric focusing was freed of low molecular weight contaminants by hydration, exhaustive washing with distilled and deionized water, and dehydration with anhydrous methanol. Other materials were as in previous work (McIntire et al., 1985).

Isolation of the Enzyme. Pseudomonas putida (N.C.I.B. 9869) was grown and harvested as before (Keat & Hopper, 1978), but a new purification procedure was devised so as to avoid the heat denaturation step and improve the yield and purity. The cells were collected by continuous-flow centrifugation at 4 °C, subjected to a freeze–thaw cycle, and suspended in 16 mM potassium phosphate buffer, pH 7.0, to give 2.5 mg of protein/mL. Per 120 g (wet weight) of cell paste used, 1 mg of deoxyribonuclease was added, and the suspended cells were disrupted by three passes through a French pressure cell at 2500 psi. Cell debris was removed by centrifugation at 39000*g* for 30 min, and the resulting supernatant solution was centrifuged at 160000*g* for 17 h. The viscous red layer formed between the pellet and the supernatant was removed and saved, and the pellet was resuspended in the same buffer and centrifuged at 270000*g* for 3 h to give a turbid supernatant solution. This solution was combined with the viscous red layer and dialyzed twice against a 15-fold excess of the same buffer. The solution (~120 mL) was applied to a DEAE-cellulose (diethylaminoethylcellulose) column (5 cm × 50 cm, Whatman Chemical Separation Ltd., DE 53) and eluted with a linear

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* Address correspondence to this author at the Veterans Administration Medical Center, San Francisco, CA 94121.

[1] Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; PCMH, *p*-cresol methylhydroxylase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Table I: Purification of *p*-Cresol Methylhydroxylase

purification step	volume (mL)	total protein (mg)	total units[a]	sp act. (μmol min^{-1} mg^{-1})	recovery (%)	purification (*x*-fold)
crude cell extract	268	11991	2949	0.25	100	1
DEAE-1	277	1047	2856	2.73	97	10.9
DEAE-2	238	424	1622	3.83	55	15.3
HTP	125	44	789	18.03	27	72.1

[a] Units are micromoles of DCIP reduced per minute in the standard PMS–DCIP assay (McIntire et al., 1985).

potassium chloride gradient (0–0.5 M) in 16 mM potassium phosphate, pH 7.0. Two peaks of activity were typically obtained, with the first peak representing approximately 20% of the total activity recovered; these were kept separate for subsequent processing. The fractions having enzyme activity under each peak were pooled, concentrated, and dialyzed against 16 mM potassium phosphate, pH 7.6, prior to being loaded onto a second DEAE column (2.5 cm × 120 cm). Elution employed a linear potassium chloride gradient (0–0.5 M) in 16 mM potassium phosphate, pH 7.6. The fractions containing enzyme activity were pooled, concentrated, and dialyzed against 16 mM potassium phosphate, pH 7.0. This concentrated solution was applied to a hydroxylapatite column (1.5 cm × 35 cm, Bio-Rad Laboratories Inc., Bio-Gel HTP) and eluted with a linear phosphate gradient (16–250 mM phosphate, pH 7.0). Fractions containing enzymatic activity were pooled, concentrated, and dialyzed against glass-distilled water prior to lyophilization and storage at −30 °C. All kinetic and subunit dissociation studies in this paper used fractions obtained from the major peak. The absorbance ratio of such fractions (A_{280nm}:A_{412nm}) was less than 1.5. A typical isolation is summarized in Table I. The enzyme isolated from the first peak in the initial DEAE-cellulose chromatography and purified by the same procedure had essentially the same specific activity, and its known properties were identical with those of the main fraction. The reason for the appearance of two peaks with activity in the elution profile of the first chromatography is not known.

Isoelectric Focusing. Resolution of the flavoprotein and cytochrome subunits was performed by a modification of the method of McIntire & Singer (1982). The ampholyte mixture of the original procedure was replaced by a small-molecule amphoteric buffer mixture described by Nguyen & Chrambach (1976). One gram of Sephadex G-200 Superfine was suspended and allowed to hydrate overnight in 34.5 mL of a solution which was 20 mM in each of the following buffers: *N*-(2-acetamido)-2-aminoethanesulfonic acid, acetic acid, aspartic acid, *N*,*N*-bis(2-hydroxyethyl)glycine, γ-amino-*n*-butyric acid, glycine, 2-(*N*-morpholino)ethanesulfonic acid, 3-(*N*-morpholino)propanesulfonic acid, propionic acid, taurine, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid, and *N*-[tris(hydroxymethyl)methyl]glycine. A portion of this slurry was then layered onto a glass plate (7.1 × 12.8 × 0.15 cm) and dried to 75% of the original weight. A concentrated solution of the native enzyme (typically 200 μL of a 20 mg/mL solution) was applied directly to the plate, which was then focused at a constant power of 7 W at 4 °C in a Pharmacia Model FBE 3000 flat-bed apparatus. The separation was complete after 2–4 h, and the final gradient was approximately linear in the range pH 3.0–6.0. The separated subunits were quantitatively eluted by repeated extraction with minimal volumes of 10 mM potassium phosphate (pH 7.60). The subunit solutions were then concentrated in an Isco isoelectric concentrator with 10 mM potassium phosphate (pH 7.60) in the low-side reservoir and 100 mM potassium phosphate (pH 7.60) in the high side (Allington et al., 1978). Typical yields were 0.5 mL of solutions which were 3 mg/mL in the subunits.

Other Methods. Unless otherwise mentioned, the buffer used for enzyme incubations was 10 mM potassium phosphate, pH 7.60, adjusted to the desired ionic strength with KCl. Activity was determined by the modified PMS–DCIP assay recently described (McIntire et al., 1985) in Tris-HCl buffer, pH 7.6 (ionic strength = 0.05 M at 25 °C), except that in monitoring column fractions fixed (2 mM) subsaturating PMS and saturating *p*-cresol (0.7 mM) concentrations were used. K_D values for the dissociation of the flavocytochrome into its subunits were determined by a "cytochrome titration" protocol wherein a fixed concentration of the flavoprotein subunit was incubated with varying concentrations of the cytochrome subunit, both subunits being either fully oxidized with potassium ferricyanide or fully (i.e., stoichiometrically) reduced with sodium dithionite under anaerobic conditions. For studies on the effects of substrates and inhibitors on the subunit dissociation, the cytochrome titration procedure was modified to include the particular reagent in the incubation mixture, typically at concentrations numerically equal to the K_M or K_I. Note that under the experimental conditions described herein the subunit dissociation constants were usually less than 100 nM; consequently, in order to cover a range of cytochrome concentrations sufficient to describe the subunit binding accurately, very low flavoprotein concentrations (~20 nM) were employed in the incubation mixture. Thus, large aliquots of the incubation mixture were needed in the activity assays to achieve rates significantly above the nonenzymatic background reaction found in the PMS–DCIP assay at pH 7.6; e.g., 300 μL of the incubation mixture was added to an assay cuvette for a final volume of 3.00 mL. For studies on the effect of temperature on the dissociation constant, if the incubation mixture were at a temperature far from the assay temperature, a resulting change in the observed rate might be seen solely as a result of the temperature change upon addition of the enzyme sample. However, such a change in temperature would be equivalent for all of the incubation mixtures for a given incubation temperature and would not change the observed dissociation constant; at worst, a minor perturbation of the apparent maximum velocity might be observed.

The double-difference spectroscopic technique used to determine the extinction coefficient perturbation upon subunit binding (see Results) employed a computer-operated Cary 14 spectrophotometer (McIntire, 1983; McIntire et al., 1985) and 3.0-mL tandem in-line quartz cuvettes with a path length of 0.5 cm for each compartment. All spectra have been corrected to a path length of 1.0 cm. Extinction coefficients used for the flavoprotein, cytochrome, and flavocytochrome were taken from McIntire et al. (1985).

Analysis of Data. Binding isotherms for PCMH flavocytochrome formation are described by a quadratic equation of the form

$$\frac{AB}{A_t} = \frac{A_t + B_t + K_d - \sqrt{(A_t + B_t + K_D)^2 - 4A_tB_t}}{2A_t} Q \quad (1)$$

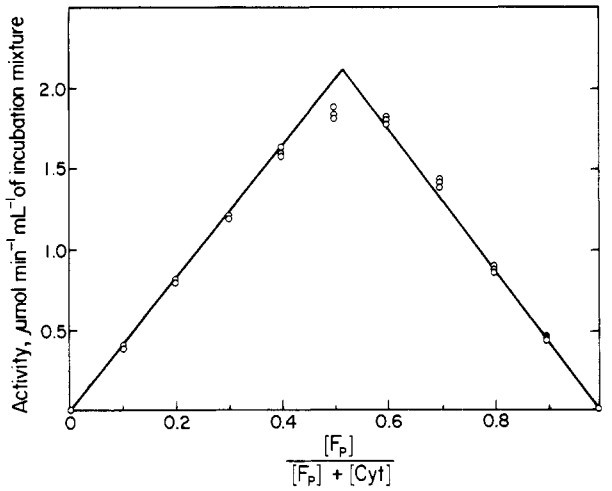

FIGURE 1: Determination of subunit stoichiometry by the method of continuous variations. Subunits prepared by isoelectric focusing were mixed in each of 11 assay aliquots so that the total protein concentration remained constant at 2.00 μM but the [flavoprotein]:[total protein] ratio varied from zero to unity. The solutions were incubated for 3 h at 25.0 °C in 10 mM phosphate buffer, pH 7.60, I = 0.200 M. Aliquots were assayed in triplicate at 25 °C in Tris–HCl, pH 7.60, I = 0.050; [PMS] = 2.00 mM, [DCIP] = 87.8 μM, and [p-cresol] = 1.67 mM. The ordinate represents the optical density change per minute per milliliter of incubation mixture at 600 nm, corrected for the small activity associated with the flavoprotein alone.

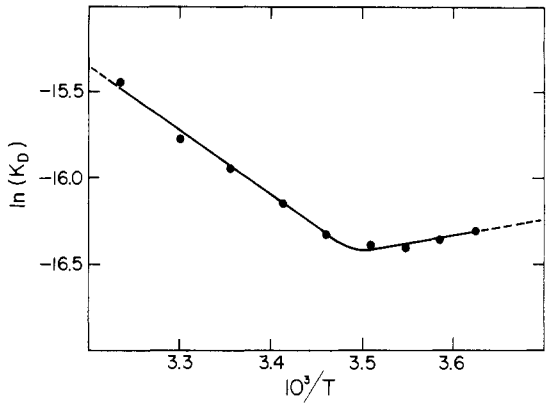

FIGURE 2: van't Hoff plot of flavoprotein–cytochrome dissociation. The cytochrome titration technique was used to determine the apparent dissociation constant for the flavoprotein and cytochrome as a function of temperature. Conditions of incubation: 10 mM phosphate, pH 7.60, I = 0.050 M, temperatures as shown in the plot. Standard assay conditions are given in the text. The approach to equilibrium was monitored by removing and assaying aliquots with time. The incubation time ranged from 10 min at 36.0 °C to 40 h at 3.0 °C.

where A_t and B_t are the initial concentrations of the subunits, K_D is the dissociation constant, and Q is some property (e.g., enzymatic activity) proportional to the concentration of the heterodimer. Data described by eq 1 were fit for the two unknowns K_D and Q by a nonlinear least-squares algorithm originally described by Bevington (1969) and modified to run on the IBM personal computer. Similar reiterative techniques were used for zero- and first-order reactions.

RESULTS

Stoichiometry of Reconstitution. In our preliminary note (McIntire & Singer, 1982), we reported that the flavoprotein subunit, isolated as described above, although free of contamination with either the cytochrome subunit or the unresolved enzyme, retains ~2% of the catalytic activity of native PCMH in the standard assay. On recombination of the two subunits, a substantial part of the original activity was recovered. As will be shown below, under appropriate conditions complete recovery of the catalytic activity may be achieved.

In studying the conditions for optimal reconstitution, several questions arose. One of these is the stoichiometry of recombination. Using catalytic activity as a measure of the degree of association of the flavoprotein and cytochrome subunits, a measurement of the stoichiometry of the species active in solution was obtained by the method of continuous variation (Job, 1927). Implicit in this experiment is the assumption that the association state of the subunits does not change upon dilution into the assay cuvette during the sampling time of the assay; this assumption is addressed below (see Dependence of K_D on Ionic Strength). Dilutions of flavoprotein and cytochrome were made into each incubation aliquot so that the total protein concentration (i.e., [flavoprotein] + [cytochrome] = [total protein]) remained constant at 2.00 μM but the [flavoprotein]:[total protein] ratio varied from zero to unity. As shown in Figure 1, a characteristic peak in activity at a molar ratio of 1:1 (flavoprotein:cytochrome) clearly implicates the heterodimer as the catalytically active species in solution. Note that the points in the figure have been corrected for a small contribution to the observed rate attributable to the free flavoprotein. The correction was made by assuming a dissociation constant of 50 nM to characterize the heterodimer formation and thus calculate the concentration of free flavoprotein at each point along the curve. Because the free flavoprotein activity under the conditions shown in the figure legend is approximately 2% that of the flavocytochrome, this correction does not appreciably change the intersection of the left- and right-side asymptotes at a value of 0.5, which implies a 1:1 stoichiometry.

Comparison of Native and Reconstituted Enzymes. K_M and V_{max} values for p-cresol and PMS were determined for the native and reconstituted enzymes in the presence of 100 μM DCIP. For the native enzyme, $K_M^{p\text{-cresol}}$ = 18.2 μM, K_M^{PMS} = 3.00 mM, and V_{max} = 53.0 ± 5.3 μmol min^{-1} mg^{-1}. For the reconstituted enzyme, $K_M^{p\text{-cresol}}$ = 19.0 μM, K_M^{PMS} = 3.06 mM, and V_{max} = 46.0 ± 4.6 μmol min^{-1} mg^{-1}. Considering the possible summation of errors in secondary plots, as used here, the values for the intact and reconstituted enzymes are in good agreement. In several other experiments of this type, the specific activities of the untreated and reconstituted enzymes agreed within 2–4%. Moreover, bisubstrate kinetic analysis of the oxidation of p-cresol and other substrates gave parallel line kinetics in steady-state experiments with the flavocytochrome, converging line kinetics with the flavoprotein, and a return to parallel line kinetics on reconstitution (McIntire, 1983; W. McIntire, D. J. Hopper, and T. P. Singer, unpublished results). Thus, the kinetic mechanism of the native flavocytochrome is restored on recombination of the subunits.

Dependence of K_D on Temperature. The effect of temperature on the apparent dissociation constant for the flavoprotein–cytochrome dissociation reaction was investigated in the range 3.0–36.0 °C. Experimental details are given in the legend to Figure 2. The apparent dissociation constant for each temperature was determined by the cytochrome titration technique described above. The data in Figure 2 are plotted as a van't Hoff plot of the natural logarithm of the apparent dissociation constant against the reciprocal of the absolute temperature. Most noteworthy is an apparent break in the curve near 15 °C. At temperatures above this value, the dissociation is characterized by a positive ΔH of 12.6 kcal mol^{-1}. Below 15 °C, the van't Hoff plot is seen to flatten out, indicating a zero or slightly negative ΔH for the dissociation. This would be consistent with a temperature-induced change in the conformational state of one (or both) subunit(s) between

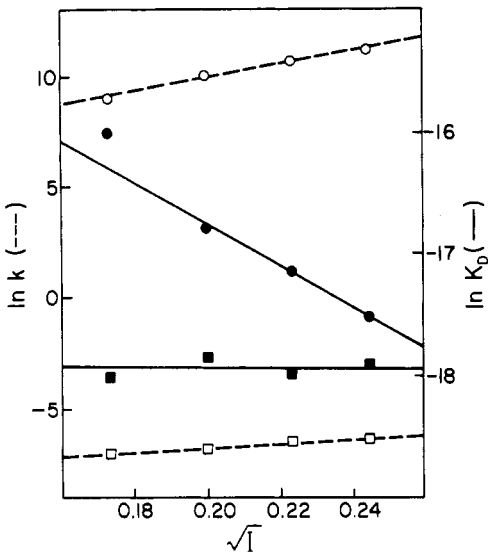

FIGURE 3: Effect of ionic strength on dissociation rates and equilibria. Debye–Hückle plot (natural logarithm of rate/equilibrium constant vs. the square root of the ionic strength) for oxidized and sodium dithionite reduced subunits. All dissociation rates are given in units of s^{-1}. Dissociation rates (open squares): In a typical experiment, a concentrated solution of PCMH (5 μM) in 10 mM phosphate buffer, pH 7.60, I = 0.100 M, 25.0 °C, was diluted to 20 nM in a buffer containing 10 mM phosphate, pH 7.60, ionic strength adjusted with potassium chloride, and the enzymatic activity was followed as a function of time by the standard PMS–CDIP assay. Dissociation constants for oxidized PCMH (closed circles): The cytochrome titration technique was used as described in Figure 2 except that the ionic strength was adjusted with potassium chloride and the temperature was fixed at 25.0 °C. Association rates (open squares): These values were not measured directly but represent the k_{off}:K_D ratio at each value of the ionic strength. Dissociation constants for reduced PCMH (closed squares): The flavoprotein and cytochrome subunits were stoichiometrically reduced with sodium dithionite under anaerobiosis, and the dissociation constant at each ionic strength was determined as described above for the oxidized case, except that oxygen was excluded until the actual assay was performed. Note that the open symbols refer to the left ordinate and that the closed symbols refer to the right ordinate. The range of ionic strength was limited on the low end (I = 0.030 M) by the need to maintain sufficient buffering capacity and on the high end (I = 0.060 M) by the extremely low values of the dissociation constants.

states having different binding affinities.

Dependence of K_D on Ionic Strength. The ionic strength dependence of the dissociation constant for subunit binding was investigated at 25.0 °C in the ionic strength range of 0.030–0.060 M for oxidized and dithionite-reduced subunits. If the activity measured in the PMS–DCIP assay is to be equated with the equilibrium concentration of the flavocytochrome, it must be shown that the approach to equilibrium is slow for both subunit association and subunit dissociation relative to the assay sampling time (typically 10–30 s). Consequently, the approach to equilibrium of the flavocytochrome upon a large dilution was also investigated. For the case of a simple bimolecular association–dissociation reaction, the apparent rate constant for approach to equilibrium upon dilution is numerically equal to the dissociation rate constant, k_{off} (Frost & Pearson, 1961). Figure 3 shows the dependence of the natural logarithm of the rate constant of dissociation and of the dissociation constant on the square root of the ionic strength, as in the Debye–Hückel equation. The data were derived from primary plots of the time course of the dissociation and of the equilibrium attained on titration of a fixed concentration of the flavoprotein with the cytochrome subunit. For comparison, Figure 3 includes the variation of the dissociation constant with ionic strength for the

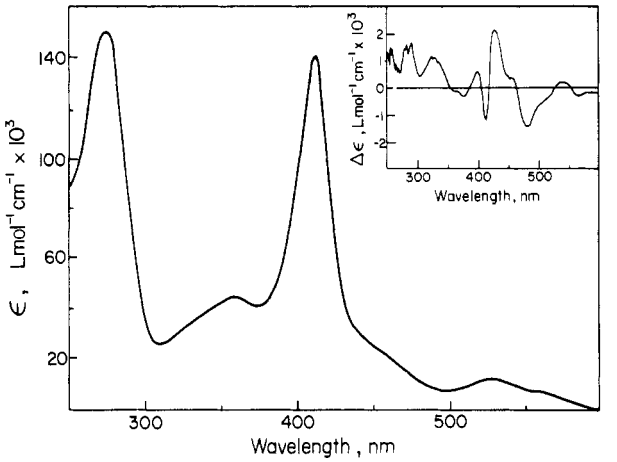

FIGURE 4: Native enzyme spectrum and difference spectrum upon reassociation. UV–visible spectrum of the flavocytochrome prior to resolution of the subunits; Tris–Tris-HCl, pH 7.60, I = 0.075 M. Inset: Difference spectrum generated by the subtraction of the separated subunit spectra (not shown) from the spectrum of the native enzyme.

dithionite-reduced subunits. Note that the ionic strength dependence of the dissociation constant is abolished in the reduced species in this ionic strength range. Also, the absolute value of the dissociation constant for the chemically reduced subunits in the ionic strength range of 0.03–0.06 M (15 nM at 25.0 °C) is nearly identical with the limiting dissociation constant found at temperatures below 15.0 °C for I = 0.05 M.

Influence of Substrates and Competitive Inhibitors on Dissociation. The flavoprotein subunit was titrated with cytochrome in the presence of *p*-cresol, *o*-cresol, and *p*-bromophenol, with both subunits stoichiometrically oxidized prior to the experiment, in order to determine the effect of substrates and substrate analogues on the K_D. Even under conditions favoring subunit dissociation (10 mM phosphate, pH 7.60, I = 0.030 M, T = 25 °C), the apparent dissociation constants were so low that their exact measurement proved impossible. Only an upper limit ($K_D \sim 5$ nM) could therefore be established.

Absorbance Changes Resulting from Recombination of the Subunits. Spectra of the isolated subunits of PCMH have already been published (McIntire & Singer, 1982). The main part of Figure 4 shows the absorption spectrum of the flavocytochrome prior to separation of the subunits. By comparison of the spectrum of the flavocytochrome with those of its constituent subunits by double-difference spectrophotometry (cf. Experimental Procedures), the difference spectrum shown in the inset of Figure 4 is observed. The mathematical manipulation used to generate this difference spectrum is a programmatic procedure available as part of the computerized controlling program for the Cary 14 (see above). As shown in Figure 4, a minor perturbation of the Soret band of the cytochrome accompanies binding of the flavoprotein and cytochrome. There are relative maxima in the difference spectrum at 398 and 438 nm and a relative minimum at 412 nm. Also, a minor perturbation of the flavin spectrum may overlap the changes in the cytochrome spectrum; there is a relative minimum at 479 nm. Note that changes in the UV region which presumably reflect intrinsic protein and adenine contributions to the difference spectrum are minor. Further, note that the maximum extinction coefficient change upon binding (2.1 mM^{-1} cm^{-1} at 428 nm) places severe constraints on the use of stopped-flow spectroscopy to monitor the association of the oxidized subunits because of the very high protein concentrations required for reasonable signal to noise levels.

Discussion

Notwithstanding the relatively high binding affinity that the oxidized flavoprotein and cytochrome subunits demonstrate, the UV–visible difference spectra described above imply that only minor tertiary changes about the prosthetic groups accompany binding. Nevertheless, the magnitude of the dissociation constant (i.e., 50 nM in the oxidized state, much lower in the reduced comparison) argues for a very high degree of complementarity between the subunits. Indeed, the flavocytochrome behaves as a single species through both the extensive purification procedures described earlier that employed a heat denaturation step (Hopper & Taylor, 1977; Keat & Hopper, 1978) and those less harsh methods used for the present work. It is particularly interesting that a method as gentle as isoelectric focusing can give completely reversible separation, offering a potentially valuable insight to the interaction dynamics of two important classes of protein, the covalently bound flavoproteins and the cytochromes.

The observation that recombination of the subunits is stoichiometric and yields the same absorption spectrum and the same steady-state kinetic parameters as seen in the native enzyme offers compelling evidence that the subunit separation and isolation procedures do not affect the integrity of either subunit. Since this is the first reported instance of the reversible resolution of a flavocytochrome, it offers a unique opportunity for studying the forces holding the subunits in the quaternary structure and for critically examining the function of each constituent protein in the flavocytochrome. As to the first of these questions, the different effects of ionic strength on K_D in the oxidized and reduced forms of the enzyme are highly suggestive. In the oxidized flavocytochrome, increasing ionic strength lowers the K_D value. In the reduced enzyme, the K_D is unaffected by ionic strength in the range examined. We interpret this to indicate that in oxidized PCMH the two subunits have the same sign of charge (negative, as judged by the results of isoelectric focusing) and that the resulting electrostatic repulsion is masked by high ionic strength. Thus, a monopole–monopole or monopole–dipole interaction is suggested. In the reduced enzyme, ionic strength no longer modulates the binding because there is not dependence of K_D on ionic strength. Thus, different noncovalent interactions between the two subunits must be responsible for the very tight binding. These interactions must be very strong and independent of charge because at pH 7.6 the net charge on both subunits is known from isoelectric focusing to be negative.

The presence of substrates and substrate analogues further reduces the K_D value. This is not due to reduction of the enzyme, since *p*-bromophenol and *o*-cresol are not oxidized (McIntire, 1983). Since the K_M for *p*-cresol is virtually identical in both the flavoprotein and the flavocytochrome (McIntire, 1983), it can be argued that the presence at the active site of para-substituted phenol (or phenolate) increases the affinity of the subunits; whether this flavocytochrome–substrate–product complex represents the most stable state in a true thermodynamic sense or is merely kinetically stabilized cannot be discerned presently. However, the observation that *p*-bromophenol similarly decreases the apparent dissociation constant argues strongly for a direct effector role.

The temperature dependence of the subunit interaction is unexpected and very interesting (Figure 2). There appear to be two states of the oxidized enzyme separated by a definite "break" in the van't Hoff plot. Above 15 °C, the dissociation is highly dependent on the temperature; however, at lower temperatures, this dependence disappears. Possibly, at the lower temperatures, the enzyme assumes a different solution conformation more conducive to tight binding.

Perhaps the most important aspect of the resolution and reconstitution of PCMH is the observation that the isolated flavoprotein subunit retains 2% of the catalytic activity in the *p*-cresol–PMS assay, while the K_M for *p*-cresol remains substantially unaltered during the cycle of dissociation and recombination (McIntire, 1983; McIntire et al., 1985), but, on recombination with the cytochrome subunit, the full catalytic activity and kinetic mechanism return (this paper; McIntire, 1983). We have reported that the low activity of the flavoprotein in the catalytic assay is not due to any contamination with cytochrome but is a fundamental property of the isolated flavoprotein, since in stopped-flow studies reduction of the flavoprotein by *p*-cresol is at least an order of magnitude slower than the corresponding reaction in the flavocytochrome (McIntire, 1983). It is therefore likely that one of the functions of the cytochrome is to modulate the reactivity of the flavoprotein; i.e., combination with the cytochrome might alter the conformation at the catalytic site, so as to increase the efficiency of the oxidation–reduction step, just as combination with the substrate appears to induce a conformation favoring very tight binding of the cytochrome. To test these hypotheses directly, we have initiated a crystallographic study of the tertiary structure of the flavocytochrome and of its subunits in their various states (Shamala et al., 1985).

Registry No. PCMH, 66772-07-4.

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