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Amino Acid and Sequence Analysis of the Cytochrome and Flavoprotein Subunits of *p*-Cresol Methylhydroxylase†

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ABSTRACT: The flavocytochrome *p*-cresol methylhydroxylase from *Pseudomonas putida* has been reported to have a M_r of 114 000 and to consist of two subunits, a flavoprotein and a cytochrome *c*, each with a M_r of 58 000. Recent X-ray crystallographic data from our laboratories [Shamala, N., Lim, L. W., Mathews, F. S., McIntire, W., Singer, T. P., & Hopper, D. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4626–4630], however, indicate an $\alpha_2\beta_2$ structure and a much lower molecular mass (~8000) for the cytochrome subunit. In this paper we report data confirming the conclusions of X-ray crystallographic analysis. From quantitative amino acid analysis, the molecular mass of the flavoprotein monomer is shown to be 48 600 ± 2200 and that of the cytochrome 8780 ± 250. These values have been confirmed by gel electrophoresis under denaturing conditions. Gel chromatography under nondenaturing conditions shows that the isolated flavoprotein exists as a dimer, whereas the isolated cytochrome is a monomer. The complete amino acid sequence of the cytochrome *c* subunit is presented and is shown to have regions of homology to other bacterial *c*-type cytochromes. The partial N-terminal amino acid sequence (56 amino acids) of the flavoprotein subunit is also reported. The implications of the now established tetrameric structure of the flavocytochrome on data in the literature regarding the redox and association properties of the subunits are examined.

p-Cresol methylhydroxylase (PCMH),[1] a flavocytochrome from pseudomonads, converts *p*-cresol anaerobically first to *p*-hydroxybenzyl alcohol and then to *p*-hydroxybenzaldehyde and catalyzes analogous reactions with longer chain *p*-alkylphenols. The enzyme has been the subject of intensive studies in recent years because of its unusual properties, e.g., the presence of a novel type of covalently bound flavin (McIntire et al., 1981), and the unique property among flavocytochromes of permitting resolution of its subunits and complete reconstitution of the native quaternary structure and catalytic properties (Koerber et al., 1985).

Keat and Hopper (1978) originally reported a molecular mass of 114 000 and concluded that the enzyme had an $\alpha\beta$ quaternary structure, consisting of one molecule each of flavoprotein and *c*-type cytochrome with identical molecular masses of ~58 000. Since there was no reason to doubt these conclusions, the $\alpha\beta$ structure was used for many years to interpret data concerning the enzyme, such as the results of

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[1] Abbreviations: PCMH, *p*-cresol methylhydroxylase; DNS, dansyl; FAD, flavin adenine dinucleotide; polybrene, 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

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laser flash photolysis studies of its kinetic mechanism (Bhattacharyya et al., 1985). The results of X-ray crystallographic studies at 6-Å resolution, however, strongly suggested that PCMH has, in fact, an $\alpha_2\beta_2$ structure, with molecular masses of ~50 000 and 8000 for the flavoprotein and cytochrome subunits, respectively. This necessitated a reexamination of the molecular mass values by independent means.

In this paper we report the results of quantitative amino acid analyses of the separated flavoprotein and cytochrome subunits and show that, in agreement with values derived from gel electrophoresis under denaturing conditions, the molecular masses are 48 600 ± 2200 and 8780 ± 250, respectively. The molecular mass of the native flavoprotein subunit was found to be 104 000 by gel filtration, consistent with the prediction that it exists as a dimer. The native cytochrome, on the other hand, behaved as a monomer during gel filtration (M_r 8700).

In order to interpret the high-resolution X-ray crystallographic studies now in progress in terms of the three-dimensional structure of PCMH, we have initiated studies of the amino acid sequences of the two subunits. This paper presents the complete sequence of the cytochrome subunit and a partial sequence of the N-terminus of the flavoprotein subunit.

The findings presented necessitated reevaluation of data in the literature regarding the events occurring during reductive titration of the enzyme (McIntire et al., 1985) and during flash photolysis in the presence of deazariboflavin (Bhattacharyya et al., 1985). As shown below, these observations may now be readily explained in terms of the $\alpha_2\beta_2$ structure of the enzyme.

Materials and Methods

Reagents. Carboxypeptidase Y, iodoacetamide, dithiothreitol, horse heart cytochrome *c* (type VI), tryptophan, glutamine, and dansyl chloride were from Sigma Chemical Co. Mercuric chloride, phosphoric acid, HPLC-grade sodium acetate, and ammonium acetate were from J. T. Baker Chemical Co. Pierce Chemical Co. supplied the sequanal-grade phenyl isothiocyanate (1-mL ampoules under N_2), ninhydrin, pyridine, triethylamine, and the HPLC/spectrograde trifluoroacetic acid, as well as a standardized solution of the common amino acids except tryptophan. Other reagents were obtained as indicated: acetonitrile HPLC grade from Burdick and Jackson Laboratories, Inc., electrophoretically pure urea from Bio-Rad Laboratories, ultrapure guanidine hydrochloride from Schwarz/Mann, DL-norleucine from Nutritional Biochemical Corp., DL-α-aminobutyric acid and ε-aminocaproic acid from Eastman Kodak Chemical Co., asparagine from Aldrich Chemical Co., 88% formic acid from MCB Manufacturing Chemists, Inc., 30% H_2O_2 from Fisher Scientific Co., phenol from Mallinckrodt Chemical Works, and [^{14}C]iodoacetamide, 13.4 mCi/mmol, from ICN Radiochemicals.

The molecular mass standards for gel permeation experiments were from Pharmacia Fine Chemicals, Bio-Rad Laboratories, and Sigma Chemical Co. The *Pseudomonas aeruginosa* cytochrome *c*-551 used in these experiments was a gift from T. Meyer, University of Arizona, Tuscon. The standards for the gel electrophoresis experiments were purchased from Bethesda Research Laboratories.

Other Materials. Micropolyamide sheets (5 × 5 cm) were from Schleicher & Schuell, Inc., the Techsphere column (0.46 × 15 cm) containing octadecylsilane-derivatized silica gel, 3-μm particle size, was from Phenomenex, the Spherogel-TSK 2000 SW and Spherogel-TSK 3000 SW gel permeation columns (0.75 × 30 cm), manufactured by Toyo-Soda, were purchased from Beckman Instruments, Inc., the Ultrogel AcA 202 gel was from LKB Instruments, Inc., and the Centricon-10 and -30 centrifuge concentrators were from Amicon Corp.

Pseudomonas putida (NCIB 9869) was grown on 3,5-xylenol and then sodium succinate, as previously described (Keat & Hopper, 1978; Hopper & Kemp, 1980). The A form of *p*-cresol methylhydroxylase (PCMH) was isolated, and the pure flavoprotein and cytochrome subunits were separated by isoelectric focusing, as in our previous studies (Koerber et al., 1985).

Processing of Proteins for Amino Acid and Sequence Analyses. The separated flavoprotein and cytochrome subunits of PCMH, were individually eluted from the Sephadex support used in the isoelectric focusing with 0.1 M sodium acetate buffer, the former at pH 5.0 and the latter at pH 4.5. The amino acid contaminants originating from the ampholyte mixture (aspartic acid and glycine) or from the anode and cathode solutions (glutamic acid and histidine, respectively) were removed from the protein as follows. The cytochrome subunit (2–3 mg in 8 mL) was concentrated 40-fold in Centricon-10 centrifuge concentrators, diluted to 2 mL with 0.1 M acetate buffer, pH 4.5, and again concentrated at least 10-fold. The latter procedure was carried out 3 times with the acetate buffer and then 4 times with 50 mM sodium phosphate–250 mM KCl, pH 7. The cytochrome was then applied to a TSK 2000 SW column and eluted with the phosphate–KCl buffer. Finally, salts were removed by 10–20-fold concentration 4 times with distilled water, in Centricon-10 concentrators. The flavoprotein (~15 mg in 8 mL) was processed by the same procedure, except Centricon-30 concentrators and a TSK 3000 SW column were used and desalting was accomplished by dialysis against distilled water.

Amino Acid Analysis and Calculation of Molecular Mass. The concentration of each protein solution was determined from the published molar extinction coefficients: horse heart cytochrome *c*, $\epsilon_{410} = 113 \times 10^3$; the cytochrome and flavoprotein subunits of PCMH, $\epsilon_{412} = 123 \times 10^3$ and $\epsilon_{440} = 11.7 \times 10^3$, respectively (McIntire et al., 1985). A precisely measured aliquot of each protein solution and of an internal standard solution containing α-aminobutyric acid, ε-aminocaproic acid, and norleucine was mixed, and an accurately measured aliquot of each mixture was dried in 10 × 70 mm ignition tubes. Samples were hydrolyzed for 25, 50, or 75 min at 166 °C in sealed tubes in vacuo in 300 μL of a 2:1 (v/v) solution of concentrated HCl–trifluoroacetic acid, containing 0.015% (v/v) phenol (Tsugita & Scheffler, 1982). Other aliquots of each solution were dried in ignition tubes and treated with 100 μL of performic acid at 0 °C for 30 min, after which the reagent was removed in vacuo and the residue hydrolyzed for 25 min as described above. It was ascertained that the standards were stable under the conditions of oxidation and hydrolysis.

Following hydrolysis, the tubes were cooled and opened, and the contents were dried under vacuum in a desiccator. Amino acids were quantitatively analyzed by a method similar to two recently published procedures (Heinrikson & Meredith, 1984; Cohen et al., 1984). First, the amino acids were converted to the phenylthiocarbamyl derivatives, by a slight modification of the procedure of Heinrikson and Meredith (1984). The derivatized amino acids were then separated at 52 °C on a 0.46 × 15 cm Techsphere column containing octadecylsilyl-derivatized silica gel, 3-μm particle size. The elution gradient was generated with two Altex 110 A pumps and an Altex 420 systems controller programmer. Solvent A consisted of either 0.14 M ammonium or sodium acetate, pH 6.15 (adjusted with H_3PO_4), and solvent B was a 60:40 (v/v) mixture of CH_3CN

and water. Detection was carried out at 254 nm with a 5-μL flow cell and an Altex B-R1A integrator. Control samples containing the same amount of internal standards as the protein samples were treated by the same procedure in triplicate (<1% deviation among samples) and the areas under the peaks compared with those of the standards in the protein samples and used to obtain a suitable correction in the calculations.

The content of each amino acid in terms of grams per mole of heme or flavin was calculated from the corrected areas thus obtained. The molecular mass of each protein included the contribution from the protoporphyrin IX and Fe(III), or FAD in the case of the flavoprotein. Control experiments involving protoporphrin IX or FAD subjected to the same procedure gave no products that altered the amino acid analysis. In order to show the reliability of the hydrolysis and analytical procedures used here, samples of horse heart cytochrome *c* were subjected to the same procedures and the results compared with those of sequence analysis and values in the literature. The agreement in both amino acid content and calculated molecular mass value was excellent.

Other Methods of Molecular Mass Estimation. A TSK 2000 SW molecular sieving HPLC column (0.75 × 30 cm) was used to determine the molecular mass values of the native flavocytochrome and of each of its subunits. The buffer used was 50 mM potassium phosphate–250 mM KCl, pH 7.0, the flow rate 1 mL/min, and the detector setting 254 nm. The following standards were used: blue dextran (M_r 2 000 000), catalase (M_r 232 000), IgG (M_r 158 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), chymotrypsinogen (M_r 25 000), myoglobin (M_r 17 000), and cyanocobalamine (M_r 1350).

The mass of the cytochrome subunit was determined by chromatography on Ultrogel AcA 202 (1 × 40 cm column; 50 mM potassium phosphate–200 mM KCl, pH 7.0, as eluant; 3.9 mL/h flow rate). The standards were *P. aeruginosa* cytochrome *c*-551 (M_r 8600), horse heart cytochrome *c*, myoglobin, and blue dextran, and detection was by absorbance at 280 and 412 nm.

Polyacrylamide gel electrophoresis of the cytochrome subunit was performed in 15% gel in the presence of 0.1% (w/v) sodium dodecyl sulfate and 6 M urea by a modification (Bethesda Research Laboratory, 1981) of the method of Shapiro et al. (1967). The molecular mass markers were ovalbumin, α-chymotrypsinogen, β-lactoglobulin (M_r 18 400), lysozyme (M_r 14 300), mammalian cytochrome *c* (M_r 13 300), bovine trypsin inhibitor (M_r 6200), and insulin (M_r 3000). Proteins were stained with Coomassie Brilliant Blue G250.

Sequence Analyses. These were performed on a Beckman 890M liquid-phase sequencer in the presence of 2 mg of polybrene. The program was 0.1 M Quadrol (Beckman 050383). Residues were identified by two independent HPLC methods (Bhown et al., 1978; Hunkapiller & Hood, 1983) and scintillation counting in the case of the [^{14}C]carboxamidomethylated protein.

Acetylation and CNBr Cleavage of Cytochrome. The pure cytochrome subunit (0.8 mg) in half-saturated sodium acetate was acetylated with acetic anhydride (Riordan & Vallee, 1967). Salts were removed by concentration several times with a Centricon-10, resuspending each time in distilled water. Only DNS-aspartic acid was detected before, but not after, acetylation when the appropriate samples were dansylated (Perham, 1978), acid hydrolyzed, and analyzed by TLC on micropolyamide sheets (Hartley, 1970). The acetylated sample was lyophilized and treated with CNBr in 70% (v/v) formic acid (Heller & Smith, 1966). At the end of the reaction, a small aliquot was again dansylated, and only DNS-Pro was detected. The sample was then lyophilized and sequenced.

Removal of Heme and Alkylation with [^{14}C]Iodoacetamide. One milligram of the cytochrome subunit was subjected to $HgCl_2$ treatment in 0.1 M HCl and 8 M urea (Bruschi & Le Gall, 1972) in order to remove the heme. After treatment with dithiothreitol and centrifugation to remove the insoluble Hg(II) complex, the sample was washed 4 times with water in a Centricon-10 concentrator. The sulfhydryl groups of the apoenzyme were then alkylated with [^{14}C]iodoacetamide in 6 M guanidine hydrochloride, pH 8.3 (Allen, 1981), and exhaustively dialyzed against water.

Sequencing of Carboxy Terminus with Carboxypeptidase Y. The cytochrome subunit (0.53 mg or 61.6 nmol) was treated with 0.75 mL of 5% (w/v) trichloroacetic acid for 10 min at 21 °C and centrifuged, and the supernatant solution was removed. To the residue 0.75 mL of 0.1 M pyridinium acetate, pH 5.5, containing ~0.2 mg of carboxypeptidase Y and 65 nmol of the α-aminobutyric acid internal standard was added, and the solution was incubated at 21 °C. Aliquots of 50 μL were removed periodically and rapidly injected into test tubes surrounded by dry ice to quench the reaction rapidly. The samples were then quickly lyophilized, derivatized with phenyl isothiocyanate, and analyzed by the HPLC method described above. Again, three samples containing only the same concentration of the internal standard were subjected to the same procedure. The average area (<±1%) was used to correct the areas obtained from the carboxypeptidase reaction.

Results

Quantitative Amino Acid Analysis of Subunits. Samples of the proteins to be analyzed were hydrolyzed with a 2:1 (v/v) mixture of concentrated HCl and trifluoroacetic acid at 166 °C for 25, 50, and 75 min. This method, like the conventional procedure in 6 N HCl at 100 °C, destroys Cys and Trp and slowly destroys Ser and Thr (Tsugita & Scheffler, 1982). Cys was determined by oxidizing the protein samples with performic acid prior to acid hydrolysis. The acid was removed from the hydrolyzed samples, and the amino acids were converted to their phenylthiocarbamyl derivatives, separated on an octadecylsilyl-derivatized silica gel HPLC column, and detected at 254 nm (Heinrikson & Meredith, 1984; Cohen et al., 1984).

Table I presents the results of analysis of the two subunits. The first column for each protein represents moles of amino acid per mole of heme or FAD. The minimum molecular mass calculated from the sum of the masses of the amino acids agrees well with the value calculated from summation of the masses derived from the integer values. While the amino acid analysis procedure used seems reliable for molecular mass determinations, there are a few discrepancies in the case of the cytochrome subunit that need comment. The values derived from amino acid analysis are lower by one residue for Asx, Cys, Glx, and Ser than those obtained from sequence data, while those for Ala and Pro are higher by one residue. This is not unexpected for Cys, since both residues are involved in a thioether linkage to the heme and cleavage may be incomplete or some Cys may be destroyed during removal of the heme. As for Ser, the value 5.33 ± 1.1 mol/mol of heme is the same within experimental error as the value of 6 obtained from sequence analysis; Ala and Pro gave ~0.5 residue higher for the amino acid analysis than did the sequence data for horse heart cytochrome *c*, in contrast to other amino acids, which showed very satisfactory agreement (data not shown).

Table I: Amino Acid Content and Molecular Mass Values of Flavoprotein and Cytochrome Subunit Monomers

	cytochrome subunit			flavoprotein subunit	
	mol/mol[a]	integer values[b]	from sequence	mol/mol[a]	integer values[b]
Ala	7.95 ± 0.07	8 (±0.07)	7	38.5 ± 0.17	38–39 (±0.5)[c]
Arg	3.12 ± 0.01	3 (±0.12)	3	20.4 ± 0.3	20 (±0.4)
Asp	5.98 ± 0.02	6 (±0.02)	5	44.0 ± 0.9	44 (±0.9)
Asn[d]			2 } 7		
Cys[d]	1.37 ± 0.13	1 (±0.32)	2	6.6 ± 0.3	7 (±0.4)
Glu	7.39 ± 0.01	7 (±0.39)	5	46.5 ± 0.5	46–47 (±0.5)[c]
Gln[d]			3 } 8		
Gly	7.92 ± 0.06	8 (±0.08)	8	41.0 ± 1.1	41 (±1.1)
His	1.91 ± 0.03	2 (±0.09)	2	8.4 ± 0.6	8 (±0.6)
Ile	1.74 ± 0.02	2 (±0.26)	2	21.2 ± 0.5	21 (±0.5)
Leu	5.99 ± 0.03	6 (±0.03)	6	28.4 ± 0.4	28 (±0.4)
Lys	4.00 ± 0.06	4 (±0.06)	4	26.7 ± 0.4	27 (±0.4)
Met	0.796 ± 0.022	1 (±0.22)	1	17.4 ± 0.2	17 (±0.4)
Phe	2.02 ± 0.03	2 (±0.03)	2	18.9 ± 0.8	19 (±0.8)
Pro	7.96 ± 0.01	8 (±0.04)	7	26.5 ± 0.5	26–27 (±0.5)[c]
Ser[e]	5.33 ± 1.10	5 (±1.10)	6	17.1 ± 3.8	17 (±3.8)
Thr[e]	1.00 ± 0.15	1 (±0.15)	1	23.4 ± 3.1	23 (±3.1)
Trp[f]	1	1	1		
Tyr	3.78 ± 0.11	4 (±0.12)	4	17.1 ± 0.3	17 (±0.3)
Val	6.92 ± 0.02	7 (±0.08)	7	40.9 ± 1.0	41 (±1.0)
M_r[g]	8800 ± 200	8790 ± 360	8870	50 000 ± 1600	49 900 ± 1700
	8760 ± 250[h]	8770 ± 430[h]		47 100 ± 2200[h]	47 300 ± 2200[h]
av	8780 ± 250	8780 ± 430		48 600 ± 2200	48 600 ± 2200

[a] The values are based on the mole content of heme in the cytochrome and of the FAD of the flavoprotein subunit, as determined from the absorbance at 412 and at 440 nm, respectively, for the oxidized native forms. The error estimates were obtained from the average of the values of the 25, 50, and 75-min hydrolyses. [b] The error is either that from the amino acid analysis or the difference between the mol/mol value and the integer value, whichever is higher. [c] The average of the two integer values was used for the molecular mass calculation. [d] Determined as aspartic acid, cysteic acid, and glutamic acid from the amino acid analysis. [e] The values and corresponding errors for serine and threonine at time zero were found by a linear regression analysis of a plot of mol/mol vs. hydrolysis time. [f] Tryptophan was destroyed during the amino acid analysis. The values given are from the sequence data. [g] The molecular mass values include the contribution from 1 mol/mol of protoporphyrin IX, 1 mol/mol of iron for the cytochrome, and 1 mol/mol of FAD for the flavoprotein. [h] Molecular mass values from a second amino acid analysis.

It is possible that the analytical method used here yields slightly higher values for Ala and Pro. Nevertheless, the agreement in molecular mass values for the cytochrome subunit obtained by different procedures (8780 ± 430 vs. 8870) is satisfactory.

Molecular Mass Determination by Other Methods. Despite the gratifying results of molecular mass determination of the monomeric forms of the two subunits by amino acid analysis, it was considered advisable to confirm the new values by other traditional methods since the tetrameric structure of the native enzyme revealed by X-ray crystallographic analysis (Shamala et al., 1986) differed considerably from earlier reports in the literature (cf. above).

Table II compares the molecular mass values of the flavocytochrome and of its subunits obtained by the different procedures in the present work and those reported in the literature (Keat & Hopper, 1978; Shamala et al., 1986). Keeping in mind that amino acid and sequence analyses and SDS gel electrophoresis give monomer molecular mass values of the subunits, whereas molecular sieving yields the molecular mass value of the subunits as they exist in the native form, the results obtained by different methods are in good agreement. Only the HPLC analysis of the cytochrome subunit on a TSK 2000 column gave a higher value, because proteins below M_r of ~20 000 are not resolved on this gel. It is clear that the flavoprotein subunit is dimeric even when separated from the cytochrome (cf. TSK 2000 gel filtration results with values for the monomer given by the other procedures). This confirms the predictions from X-ray crystallography at 6-Å resolution (Shamala et al., 1986), indicating a large contact surface between the two flavoprotein subunits in the intact flavocytochrome. The retention of the quaternary structure of the flavoprotein after dissociation from the cytochromes is thus not surprising. X-ray crystallography also indicates that

Table II: Determinations of Molecular Mass of Flavocytochrome and Its Isolated Subunits

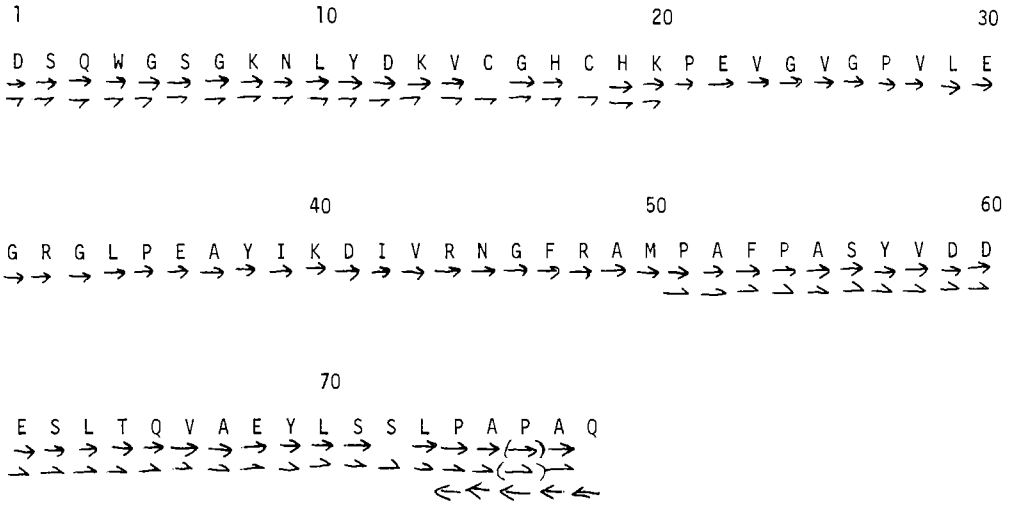
method[a]	cytochrome subunit	flavoprotein subunit	flavocytochrome
amino acid analysis	8 780 ± 250	48 600 ± 2200	
sequence analysis	8 870		
TSK 2000 gel filtration (HPLC)	~13 000	104 000	116 000
gel filtration	8 600[b]		108 000[c]
ultracentrifugation			114 000[c]
X-ray crystallographic studies[d]	8 000	50 000	119 000
SDS gel electrophoresis	6 000–7 000	58 000[c]	

[a] All proteins were in the native form except in the gel electrophoresis. [b] Ultrogel AcA 202 was used. [c] From Keat and Hopper (1978). The gel filtration involved Sephadex G-200. [d] The subunit molecular mass was estimated from a balsa wood model constructed from the 6-Å structure of the flavocytochrome (assumed M_r 116 000). The molecular mass for the flavocytochrome was measured by flotation of the crystals in a bromobenzene–xylene density gradient (Shamala et al., 1986).

the two cytochrome subunits are spatially separated from each other (Shamala et al., 1986), and the finding of a native monomeric form during gel filtration on Ultrogel AcA 202 further shows that no dimerization occurs in solution. Taken together, these data confirm the conclusion from X-ray crystallography that the native enzyme has an $\alpha_2\beta_2$ structure.

Sequence Analysis of Cytochrome and Flavoprotein Subunits. Since high-resolution X-ray crystallographic data for PCMH and its subunits are anticipated in the near future, it has become necessary to determine the primary structure of the two subunits as a requisite for deducing the secondary and tertiary structure from X-ray analysis.

Chart I: Sequencing Strategy for Cytochrome Subunit[a]



[a] The symbols →, ⇀, ⇁, and ← indicate the sequence obtained from 75 nmol of the intact protein, 8 nmol of the [^{14}C]carboxamidomethylated apocytochrome, 57 nmol of the N-acetylated/CNBr-cleaved sample, and 62 nmol of the protein treated with carboxypeptidase Y, respectively.

The cytochrome subunit was sequenced by automated Edman degradation and the results are summarized in Chart I. In order to verify the sequence at the C-terminal end, the protein was acetylated to block the N-terminal Asp and then cleaved with CNBr at the methionine in position 50, producing a mixture of two peptides, only one of which could undergo N-terminal Edman degradation. Chart I also includes the result of sequencing the mixture. The location of the cysteine residues linked to the heme was established by treatment with $HgCl_2$ in acid to remove the heme, followed by alkylation of the resulting free sulfhydryl groups with radioactive iodoacetamide. The labeled protein was sequenced, and the location of the cysteines was detected by their radioactivity. The results are also included in Chart I.

To further verify the sequence of amino acids at the C-terminus, digestion of the cytochrome subunit with carboxypeptidase Y was also studied. Figure 1 presents the time course of the release of several amino acids. Ala, Pro, and Gln were released most rapidly, but the kinetic data do not permit conclusions about the sequence of hydrolysis of these amino acids.

On continued incubation with carboxypeptidase Y beyond 3 h, a second linear phase for the appearance of Ala, Pro, and Gln is evident (Figure 1). Extrapolation of this linear phase to zero time indicates that during the rapid phase Ala, Pro, and Gln were liberated in the ratio of 2:2:1 mol/mol of heme. Along with these three amino acids, a significant release of Leu and Ser was also observed, so that at 3 h the ratio of free Ala:Pro:Gln:Leu:Ser was 2:2:1:0.18:0.16. With the exception of Gln, these data confirm the C-terminal sequence obtained by Edman degradation, which showed the sequence from residue 65 to residue 77 to be Gln-Val-Ala-Glu-Tyr-Leu-Ser-Ser-Leu-Pro-Ala-Pro-Ala. Since the Gln nearest the C-terminus is at position 65, its rapid release is unexpected. We conclude, therefore, that the C-terminal amino acid is Gln and that it was not detected during the sequence analysis.

When carboxypeptidase action was followed beyond 4 h, several other amino acids were released. The finding of significant levels of Phe and Met was surprising, since the closest Phe residue to the C-terminus is at position 53 and the single methionine in the protein is at position 50. It seems likely that the appearance of these amino acids indicated internal cleavage of the peptide chain, possibly by the action of contaminating peptidases. It has been reported (Hayashi, 1976) that carboxypeptidase Y is usually contaminated with small amounts of aminopeptidase and endopeptidase.

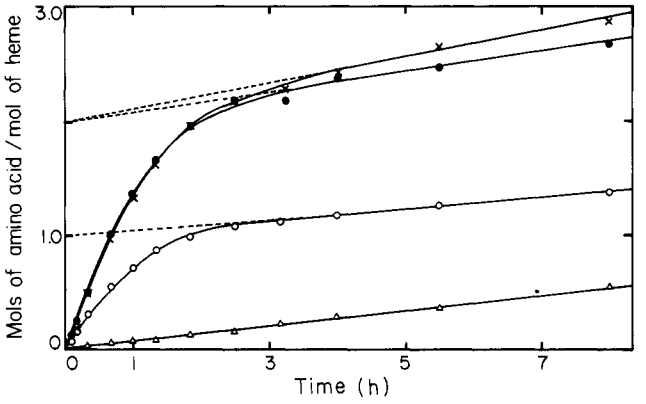
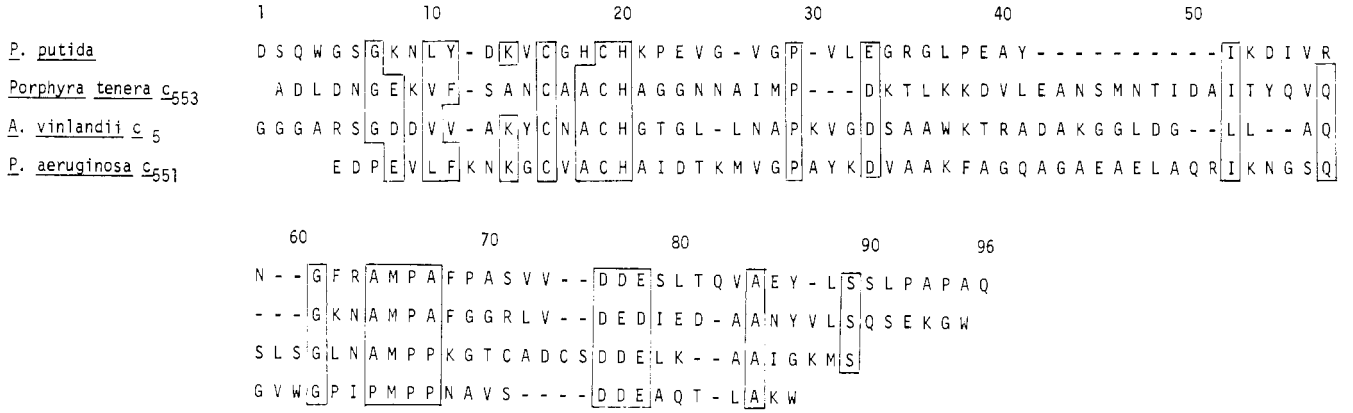


FIGURE 1: Time course for release of amino acids from denatured cytochrome subunit by carboxypeptidase Y. The moles of each amino acid released per mole of heme was determined by HPLC as described under Materials and Methods and is plotted on the ordinate. The symbols ×, ●, ○, and △ represent the data for Ala, Pro, Gln, and Leu, respectively. The dashed lines represent the extrapolation of the slow linear phase to zero time. Note that the data for Ser (not shown) are nearly identical with that for Leu.

The cytochrome subunit sequence was compared to sequences of other cytochromes found in the National Biomedical Research Foundation data bank and showed the greatest homology to *Porphyra tenera* cytochrome *c*-553 (Chart II). An alignment can be created that shows significant regions of homology between PCMH and three different cytochromes. The regions of highest homology center around the heme binding site and the heme iron ligands (Meyer & Kamen, 1982).

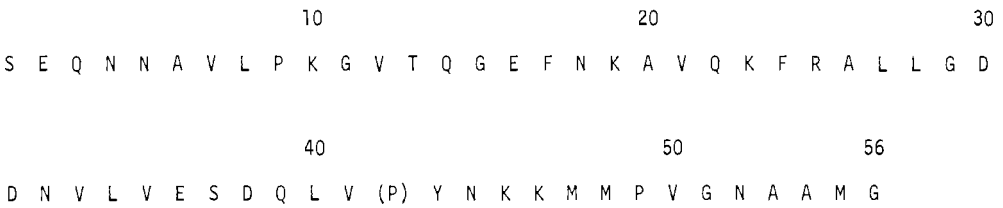
The size of the flavoprotein subunit precluded complete sequencing in one run, so that it seemed logical to derive the amino acid sequence from the DNA sequence. One purpose of the automated Edman degradation, the results of which are shown in Chart III, was to identify a sequence that would be adequate to generate a synthetic oligonucleotide probe as an aid to obtaining the PCMH gene. Chart III presents the sequence of the first 56 residues at the N-terminus with a single ambiguity at position 42. Two regions (residues 16–20 and 43–48) appear suitable to produce oligonucleotides for DNA hybridization experiments.

Chart II: Sequence Comparison of Cytochrome Subunit with Other Bacterial Cytochromes[a]



[a] The boxed regions indicate amino acids in each sequence that are identical or highly conserved when the cytochrome subunit is used as the standard.

Chart III: N-Terminal Sequence of Flavoprotein[a]



[a] The sequence was obtained from 24 nmol of the protein.

A second reason for undertaking this partial Edman degradation was to ascertain whether a sequence corresponding to the flavin binding site is present in this region. We have previously shown (McIntire et al., 1981) that the amino acid sequence around the covalently linked FAD is

FAD
|
Tyr-Asn-Trp-Arg-Gly-Gly-Gly (Gly, Ser, Met)

As seen in Chart III, no similar sequence is present at the N-terminal end of the flavoprotein.

Discussion

In most investigations prior to undertaking amino acid and sequencing analysis, the molecular masses of a protein and of its subunits are known, or at least, close estimates are available. The amino acid analysis is then adjusted to best fit these values of the molecular mass. This was not the approach taken in the present study of PCMH, for which the molecular masses of the subunits were not assumed. Although in a previous study the molecular mass of the flavocytochrome has been measured by gel filtration on Sephadex G-200 and by ultracentrifugation and of its subunits by SDS–polyacrylamide gel electrophoresis (Keat & Hopper, 1978), recent X-ray crystallographic data at 6-Å resolution (Shamala et al., 1986) cast doubts on the earlier estimates and the $\alpha\beta$ structure they implied.

Quantitative amino acid analysis, using the rapid hydrolysis and HPLC procedures described under Materials and Methods, not only yields the most accurate value but is also fast and convenient, taking ~2 h for completion for a single analysis, which involves a 25-min hydrolysis. The applicability of the method for accurate determination of the molecular mass of subunit monomers depends on the availability of reliable and convenient methods for determining the concentration of the protein in the sample to be analyzed. This is clearly the case with both the flavoprotein and cytochrome subunits of PCMH, which were readily quantitated by chemical determination of their covalently bound prosthetic groups (McIntire et al., 1985). Since the method *does not* assume prior values of the molecular masses and yields molecular mass values per mole of prosthetic group (or other chemical marker), it is essential to know the number of the markers per mole of subunit or to check the calculated molecular mass values of the monomer against estimated values derived from other methods. In the present study, both traditional methods (gel filtration, SDS gel electrophoresis, etc.) and amino acid sequencing were also used to establish these values for the subunits (Table II).

In the studies of Keat and Hopper (1978), the molecular mass of the subunits was determined by SDS electrophoresis on 6.4% (w/v) polyacrylamide gel in the presence of SDS. Only a single band, with an M_r of 58 000, was detected after staining with Coomassie Blue. In the same study, the molecular mass value of the native flavocytochrome was found to be in the range from 108 000 to 114 000, and the authors concluded that the two subunits had identical molecular mass values and that the quaternary structure was $\alpha\beta$. In this study, the isolated cytochrome subunit was analyzed on 15% (w/v) polyacrylamide gel in the presence of SDS. The molecular mass value found was 6000–7000 (Table II), and the protein gave a more diffuse band than the standards. It seemed likely that in the earlier experiments (Keat & Hopper, 1978) the cytochrome band escaped detection either because it has a poor staining property, because it ran off the 6.4% (w/v) gel, or because it was washed off prior to staining.

In the intervening years, the 1:1 ratio of the two subunits has been confirmed from several lines of evidence, including chemical determination of the prosthetic groups in the native enzyme, reductive titrations with *p*-cresol or dithionite (McIntire et al., 1981, 1985), separation and reconstitution of the subunits, and computer simulation of the flavocytochrome spectrum from the spectra of the separate subunits (Koerber et al., 1985). The molecular mass of the flavo-

cytochrome has also been confirmed by HPLC gel filtration and X-ray crystallographic studies at 6-Å resolution (Table II). The latter indicates an $\alpha_2\beta_2$ tetramer consisting of two large and identical core proteins and two separate, smaller, iron-containing domains—one bound to each of the large subunits (Shamala et al., 1986). The present study fully confirms this visualization.

In light of the information presented, showing that PCMH has an $\alpha_2\beta_2$ rather than $\alpha\beta$ structure, some of the interpretations in the recent literature on this enzyme need to be revised. An example is the behavior of PCMH during reductive titration with *p*-cresol (McIntire et al., 1985). Addition of a substoichiometric amount of substrate produced only reduced cytochrome without any sign of a flavin radical. Since the *p*-cresol is an obligatory 2-e^- donor and the heme a 1-e^- acceptor and assuming an $\alpha\beta$ structure reported in the literature (Keat & Hopper, 1978), an intermolecular mechanism was proposed, involving the reduction of ferricytochrome of one molecule by the ferrochrome of the other. Clearly, the presence of two cytochromes in each PCMH molecule makes intramolecular electron transport feasible and the more likely interpretation. In the same study it was shown that after all the heme was reduced addition of further amounts of *p*-cresol produced a flavin radical very rapidly, which then disproportionated only slowly (McIntire et al., 1985). In terms of the $\alpha\beta$ structure, again intermolecular electron transfer was invoked as an explanation. The tetrameric structure also eliminates the need for this interpretation.

The tetrameric structure of PCMH also helps to rationalize the results of flash photolysis studies in the presence of deazariboflavin (Bhattacharyya et al., 1985). The experiments reported showed the rapid production of reduced heme and of neutral flavin semiquinone radical in 2:1 ratio by the reduced deazariboflavin, followed by a slower electron transfer from the flavin to the heme. The process was shown by these workers to be intramolecular because the rates were independent of enzyme concentration.

Another set of observations that may now be reinterpreted concerns the reconstitution of the flavocytochrome from its subunits (Koerber et al., 1985). On separation of the subunits by isoelectric focusing, the flavoprotein retains only 2% of the catalytic activity on *p*-cresol at V_{max}. On recombination of the subunits, all of the original catalytic activity reappears. This permits monitoring the recombination of the subunits by steady-state assays. Kinetic and thermodynamic data on the recombination fit well the then accepted structure, so that the reaction was visualized as $\alpha + \beta \rightleftarrows \alpha\beta$ (Koerber et al., 1985). The now established $\alpha_2\beta_2$ structure permits visualization of the reaction sequence as follows. The isolated flavoprotein exists as a dimer (α_2, cf. above), while the isolated cytochrome is monomeric (β). Recombination of the two cytochromes with the flavoprotein dimer appears to be independent of each other, so that an $\alpha_2\beta$ structure with half of the catalytic activity of the native $\alpha_2\beta_2$ species may exist. This interpretation fits well the data of Koerber et al. (1985).

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