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Protein Components of a Cytochrome P-450 Linalool 8-Methyl Hydroxylase*

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The cytochrome P-450 heme-thiolate monooxygenases that hydroxylate monoterpene hydrocarbon groups are effective models for the cytochrome P-450 family. We have purified and characterized the three proteins from a P-450-dependent linalool 8-methyl hydroxylase in *Pseudomonas putida* (incognita) strain PpG777. The proteins resemble the camphor 5-*exo*-hydroxylase components in chemical and physical properties; however, they show neither immunological cross-reactivity nor catalytic activity in heterogenous recombination. These two systems provide an excellent model to probe more deeply the heme-thiolate reaction center, molecular domains of substrate specificity, redox-pair interactions, and the regulation of the reaction cycle.

Microbial catabolism of mono- and sesquiterpenoid hydrocarbons has focused primarily on reaction pathways (1–3), *e.g.* acyclic (4–6), alicyclic (7–9), and analogous aromatic structures (6). The initial hydroxylation of a methyl or methylene group provides substrates poised for catabolic pathways yielding carbon and energy for growth (15).

Due to the facility with which cytochrome P-450 monooxygenases catalyze the stereo- and regiospecific incorporation of an oxygen atom into "inert" hydrocarbon groups, the activation of terpenoid substrates is frequently P-450-mediated (9–11). These heme-thiolate proteins are broadly distributed in both pro- and eucaryotes (12–14) and share remarkable similarities in electronic and resonance spectral properties (12–16). Despite these similarities, P-450s are often extremely specific in substrate selection and electron transfer protein requirements. The selectivity of the substrate binding sites and requirements for a specific redox partner displayed by these monooxygenases are areas requiring further clarification.

The camphor 5-*exo* hydroxylase (EC 1.14.15.1) isolated from *Pseudomonas putida* PpG786 (ATCC 29607) has provided a relevant model for the P-450 family through extensive physical and chemical characterization (15–20). It has also led the studies of P-450 structure and reaction mechanism, yielded primary structures via a combination of protein and DNA techniques (21–25), and a tertiary structure by x-ray analysis at 1.6-Å resolution (26, 27). Intermediate reaction states have been characterized by a variety of chemical and spectral techniques (15–20). The operon array and regulation have been documented (23–25).

This paper reports the purification and characterization of a second soluble P-450 monooxygenase. The linalool 8-hydroxylase, first detected in collaboration with P. K. Bhattacharyya, has been purified from the strain of *P. putida* (incognita) isolated by Madyastha *et al.* (6). The monooxygenase catalyzes, in two reaction cycles, the formation of 8-oxolinalool via the 8-alcohol. The component proteins of the linalool 8-hydroxylase are similar in general properties to those of the camphor-5-*exo*-hydroxylase, but little activity is obtained in heterogenous reconstitutions. Differences in the active site environments of the two P-450s have been indicated in preliminary publications (24, 54–56).

MATERIALS AND METHODS[1]

RESULTS AND DISCUSSION

P. putida (incognita) strain PpG777 (formerly known under work number PH651), when grown on a phosphate ammonium salt (28) basal medium with (±)-linalool as the sole carbon and energy source, produces a P-450-dependent monooxygenase that catalyzes the first step in the catabolism of linalool. The whole cell content of LIN[2] P-450 reaches 2.5 mg of P-450/g of cells, wet weight, about 60% of the level of $P\text{-}450_{cam}$ produced by *P. putida*, strain PpG786. Both strains release substantial amounts of the soluble protein components upon freeze-thaw autolysis, allowing ready purification on a large scale.

Protein Purification—The purification protocols detailed in the methods were derived from the procedures established for the CAM system (28) and are equally suited for the LIN and CAM components. Principal changes in the procedure for the reductase include use of the second DEAE ion exchange column before the Bio-Gel P-100 filtration, eluting the third DEAE column with a step gradient, and the addition of two affinity columns (Affi-Gel Blue and Matrix Gel Blue A) as the final steps in the purification. The redoxin purification

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[1] The "Materials and Methods" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: LIN, linalool; CAM, camphor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

TABLE I

Pseudomonas (LIN/CAM) reductase purifications

Purifications were normalized to 1 kg cell paste.

Step	Volume	Protein	Absorbance ratio[a]	Activity[b]	Yield
	ml	*g*		*units*	%
Extract	2670/1690	161/90		42/620	100
DEAE-I	900/430	90/6.2		35/410	83/66
$(NH_4)_2SO_2$, 36–65%	100/90	75/5.5		34/380	81/61
DEAE-II	180/	89/		32/	76/
Bio-Gel P-100	220/270	15/2.5		29/340	71/55
DEAE-III	235/490	2.8/0.5[c]	/10	17/210	41/34
Affi-Gel Blue	180/	0.3/	18.0/	15/00	37/
Matrix Gel Blue A	55/360	0.02[c]/0.21[c]	8.0/7.4	14/115	34/19

[a] Determined in 50 mM Tris·Cl, pH 7.4, 5% glycerol (v/v); A_{275}/A_{456}, oxidized state.
[b] Unit = 1 nmol of NADH oxidized/s.
[c] E_{454} = 10 mM^{-1} cm^{-1} for M_r = 43,500.

TABLE II

Pseudomonas (LIN/CAM) redoxin purifications

Purifications were normalized to 3 kg cell paste.

Step	Volume	Protein	Absorbance ratio[a]	Redoxin	Yield
	ml	*g*		*g*	%
Extract	4040/3850	310/240		0.85[b]/1.8[b]	100
DEAE-I	830/1400	5.6/10.5	0.03/0.08	0.60[b]/1.4[b]	71/78
$(NH_4)_2SO_4$, 0–85%	53/70	3.7/5.5	0.05/0.46	0.49[b]/1.35[b]	58/75
Bio-Gel P-30 (P-100)	295/450	1.1/1.5	0.12/0.59	0.42[b]/0.90[b]	49/50
DEAE-II	204/330	0.34/0.65	0.90/0.71	0.34[c]/0.65[c]	39/36

[a] A_{324}/A_{280}, oxidized state, in 50 mM Tris·Cl, pH 7.4, 10 mM 2-mercaptoethanol.
[b] Estimated from activity measurements.
[c] Estimated with E_{412} = 11.3 mM^{-1} cm^{-1} (LIN), 11.1 mM^{-1} cm^{-1} (CAM), M_r = 11,600.

TABLE III

Pseudomonas (LIN/CAM) cytochrome P-450 purifications

Purifications were normalized to 3 kg cell paste.

Step	Volume	Protein	Absorbance ratio[a]	Cytochrome P450	Yield
	ml	*g*		*g*[b]	%
Extract	3730/3850	260/240	0.04/0.05	3.4/6.0	100
DEAE-I	985/1100	76/17	0.07/0.30	2.8/4.5	82/75
$(NH_4)_2SO_4$, 36–60%	180/90	47/12	0.12/0.77	2.6/4.2	76/70
Bio-Gel P-100	130/480	5.3/5.5	0.66/1.05	1.7/3.3	50/55
DEAE-II	330/420	1.3[c]/2.3[c]	1.42/1.45	1.1/2.1	32/33

[a] In 50 mM K-P$_i$, pH 7.0, 100 mM KCl, 200 μM linalool or camphor; A_{391}/A_{280}, substrate-saturating oxidation state.
[b] $E_{448\text{-}490}$ = 93 mM^{-1} cm^{-1} for $m^{rs}_{co} - m^{rs}$; M_r = 45,000 (28).
[c] E_{280} = 63 mM^{-1} cm^{-1}.

was modified by replacing Bio-Gel P-100 with Bio-Gel P-30 for the filtration step. The procedure for purification of the P-450 component remains essentially unchanged. All three protocols include minor changes in buffer composition.

The data in Tables I–III show the results of typical purifications of LIN reductase, redoxin, and P-450. For comparison the data for the purification of the corresponding CAM components are included in the tables. The data have been normalized to standard weights of cell paste (1 kg for the reductase procedure; 3 kg for the redoxin and P-450 components). Due to the high spectral background, purifications of the reductase and redoxin were monitored by NADH oxidase activity (see "Materials and Methods"). The P-450 purification was monitored by specific content (nmol of P-450/mg of protein) determined from the $Fe^{II}CO\text{-}Fe^{II}$ difference spectrum (28). Lin P-450 and redoxin levels are ~50% of their CAM system counterparts, while LIN reductase is present at ~7% of the level for CAM reductase. Whether this difference arises from levels of expression or enzyme stability is unknown. Purification of LIN reductase shows an increased yield (2-fold) relative to the CAM system. Most of this difference arises with the first DEAE column and the Matrix Gel Blue A column (Table I). Yields of CAM and LIN redoxin and P-450 are essentially equivalent (Tables II and III). The purification achieved with each step varies for the two systems due to differences in the content of the enzymes and the background in cell extracts from the two bacterial strains.

Criteria of Purity—Purity of the proteins in the final pools was estimated from SDS-PAGE and correlated with characteristic spectral parameters of the holoenzymes. LIN reductase with A_{275}/A_{456} = 8.5 was estimated to be >85% homogenous in total protein; LIN redoxin with A_{324}/A_{280} = 0.90 was >90% pure; and LIN P-450, in the presence of saturating substrate, had A_{392}/A_{280} = 1.43 and was >90% homogenous.

Storage and Stability—Following ultrafiltration or dialysis, the concentrated LIN redoxin reductase (0.16 mM), LIN redoxin (1 mM), and LIN P-450 (1 mM) solutions were packaged in 3-ml aliquots and stored in liquid nitrogen. The enzyme stocks responded differently to storage and freeze-thaw cycles. LIN redoxin reductase and LIN P-450 demon-

TABLE IV

Pseudomonas flavin (FAD) reductase properties

Property	LIN Reductase	CAM Reductase
Molecular weight, M_r		
Analytical data	43,700[a]	42,700[a,b]
SDS-PAGE	44,000	47,000[c]
Gel filtration	45,000	45,000[c]
Isoelectric point, pI		
Oxidized	4.66	4.38[c]
UV-visible,[c] nm/mM		
Oxidized	275, 381, 456, 480, 72, 9.2, 10.0, 8.3	275, 378, 454, 480, 72, 9.7, 10.0, 8.5
Turnover number,[d] s^{-1}		
Homologous	43	25
Heterologous	ND[e]	2
Immunological reaction[f]		
Homologous	+++	+++
Heterologous	−	−

[a] Determined from amino acid composition and FAD group.
[b] Tsai *et al.* (52).
[c] Gunsalus and Wagner (28).
[d] Substrate-stimulated NADH oxidation using excess amounts of redoxin and P-450 from the native (homologous) or related (heterologous) *Pseudomonas* system.
[e] Not detected.
[f] Rabbit antibody raised against reductase from native (homologous) or related (heterologous) *Pseudomonas* system.

TABLE V

Pseudomonas iron-sulfur (Fe_2S_2) redoxin properties*

Property	LIN redoxin	CAM redoxin
Molecular weight, M_r		
Analytical data	12,800[a]	11,594[a,b]
SDS-PAGE	10,700	11,700[c]
Gel filtration	11,000	11,000
Isoelectric point, pI		
Oxidized	<4.5 forms apoprotein	<4.5 forms apoprotein
UV-visible,[c] nm/mM^{-1} cm^{-1}		
Oxidized	277, 280, 324, 412, 455, 17.8, 17.5, 15.7, 11.3, 9.9	277, 280, 328, 410, 455, 22.7, 21.9, 15.6, 11.1, 10.4
EPR,[d] *g* values		
Reduced	2.021, 1.938	2.022, 1.938
Turnover number,[e] s^{-1}		
Homologous	2.0	2.0
Heterologous	ND[f]	ND

[a] Determined from amino acid composition and Fe_2S_2*Cys_4 group.
[b] Amino acid sequence, Tanaka *et al.* (53).
[c] Gunsalus and Wagner (28).
[d] 50 mM Tris, pH 7.4, 10 mM 2-mercaptoethanol, 25 °C; X-band measurements at 7 K, dithionite-reduced.
[e] Substrate-stimulated NADH oxidation using excess amounts of reductase/P-450 from the native (homologous) or related (heterologous) *Pseudomonas* system.
[f] ND, Not detected.

strated no measurable loss of enzymatic activity from prolonged storage at −196 °C nor from repeated freeze-thaw cycles. LIN redoxin, however, loses the prosthetic group during standing at 0 °C in the absence of an active reducing agent, as evidenced by the decrease in absorbance in the iron sulfide chromophore; reagent grade dithiothreitol (5 mM) was found to stabilize the redoxin by retarding apoprotein formation.

Physical and Chemical Characteristics—Pertinent physical and chemical characteristics for the three components of the LIN monooxygenase are reported in Tables IV-VII; properties of the CAM system enzymes are included for comparison. In addition to similarities of the three LIN proteins and their

TABLE VI

Pseudomonas cytochrome P-450 (heme-thiolate) properties

Property	LIN P-450	CAM P-450
Molecular weight, M_r		
Analytical data	44,800[a]	46,820[a,b]
SDS-PAGE	47,000	50,500[c]
Gel filtration	45,000	44,000[c]
Isoelectric point, pI		
Oxidized, m^{os}	4.84	4.59[c]
UV-visible, nm/mM^{-1} cm^{-1}		
Oxidized		
m^{o}	280, 360, 417, 538, 569, 63.9, 30.6, 102, 10.5, 11.2	280, 360, 417, 535, 569, 68.3, 36.7, 115, 11.6, 11.9
m^{os}	280, 392, 510, 540, 646, 62.5, 92.0, 11.7, 9.9, 4.9	280, 391, 510, 540, 646, 63.3, 102, 13.0, 11.2, 5.4
Reduced		
m^{r}	411, 540, 75.6, 13.6	480, 540, 76.7, 15.1
m^{rs}_{co}	411, 542, 73.7, 12.9	409, 542, 86.5, 16.0
m^{rs}_{co}	447, 550, 120, 17.2	446, 550, 120, 14.0
$m^{rs}_{co} - m^{rs}$	446–490, 84.0	446–490, 92.8
EPR, *g* values		
Oxidized, m^{0}	1.91, 2.27, 2.44, 8.13, 3.56, 1.68, 1.98, 2.25, 2.41	1.91, 2.26, 2.45, 7.85, 3.96, 1.77, 1.97, 2.24, 2.41
K_D (substrate),[c] μM		
Oxidized	3.6	2.9[c]
Turnover number,[d] s^{-1}		
Homologous	32	34
Heterologous	1.2	ND[e]
Immunological reaction[f]		
Homologous	+++	+++
Heterologous	−−−	−−−

[a] Determined from amino acid composition and protoheme IX group.
[b] Amino acid sequence, Haniu *et al.* (21, 22, 24).
[c] Gunsalus and Wagner (28), CAM P-450 only.
[d] Substrate-stimulated NADH oxidation using excess amounts of redoxin/reductase from the native (homologous) or related (heterologous) *Pseudomonas* system.
[e] Not detected.
[f] Rabbit antibody raised against P-450 from native or related (heterologous) *Pseudomonas* system.

CAM counterparts in chromatographic behavior, there is also marked similarity in size, chemical composition, and spectroscopic properties. SDS-PAGE and gel filtration data indicate molecular weights of reductase (44,000) redoxin (11,000), and P-450 (46,000) very close to the values for the CAM hydroxylase components. The isoelectric points for the LIN reductase and P-450 were, respectively, 4.66 and 4.84, showing both to be less acidic than the corresponding CAM proteins. These are in agreement with the amino acid compositions of the proteins. The LIN reductase has 8 more basic residues than CAM reductase (His + Lys + Arg) and probably less acidic residues (Asx + Glx, −12; amide content unknown). Similarly, the LIN P-450 has 4 more basic residues and less acidic residues (Asx + Glx, −5) than P-450$_{cam}$ (Table VII). As with CAM redoxin, the LIN iron sulfide protein loses the prosthetic group to acid decomposition (pH < 4.5) before reaching its isolectric point.

Despite the physical and chemical similarity of the two systems, the P-450s lack cross-reactivity in immunodiffusion assays with antisera raised against the purified proteins (Tables IV and VI).

Enzymatic Activity—Catalytic properties for each of the LIN monooxygenase components and the corresponding

TABLE VII
Amino acid composition of LIN/CAM monooxygenase proteins

	Reductase		Redoxin		Cytochrome P-450	
	LIN	CAM[a]	LIN	CAM[b]	LIN	CAM[c,d]
Asx	28	40(15)[e]	10	13(3)[e]	40	35(13)[e]
Thr	16	20	4	5	14	20
Ser	28	18	9	7	13	24
Glx	40	40(24)[e]	15	10(5)[e]	46	56(23)[e]
Pro	13	18	7	4	26	30
Cys	6	6	4	6	5	8
Gly	46	33	12	8	32	25
Ala	47	48	9	9	30	31
Val	40	34	7	14	32	24
Met	4	6	3	3	6	10
Ile	20	24	6	6	17	26
Leu	39	42	15	6	45	42
Tyr	11	6	1	3	7	9
Phe	10	10	4	1	19	18
His	10	6	2	2	10	13
Lys	7	13	1	3	12	12
Arg	34	24	7	5	33	26
Trp	3	3	1	1	6	5
	402	391	117	106	393	414

[a] Tsai *et al.* (52).
[b] Tanaka *et al.* (53).
[c] Haniu *et al.* (21, 22).
[d] Corrected via cloned *camC* DNA.
[e] Values in parentheses are, respectively, Asn or Gln.

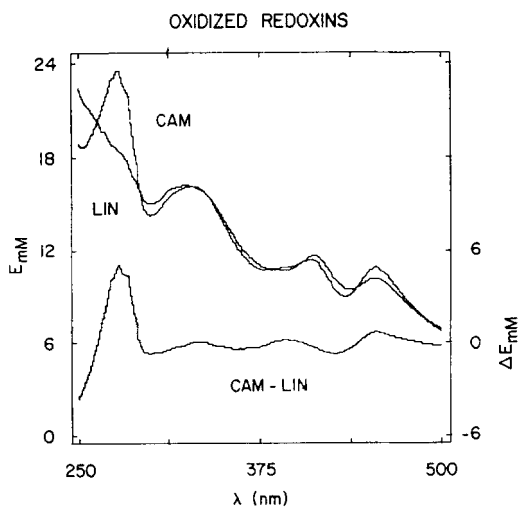

FIG. 1. **Absorption spectra of oxidized $Fe_2S^*_2Cys_4$ proteins.** Conditions as specified in Table V and under "Materials and Methods."

CAM proteins are summarized in Tables IV–VI. Each turnover number (nmol of NADH oxidized/s/nmol of enzyme) was determined in reconstitutions with the other system components in saturating excess. The P-450 and redoxin components from each system are equivalent, while the LIN reductase turnover number is 1.7 times that of its CAM counterpart (Table IV).

While the similarity in chemical, physical, and catalytic properties between the components of the LIN and CAM monooxygenases suggests similar structures, little activity is observed in assays using heterologous reconstitutions (Tables IV–VI). CAM reductase couples with LIN P-450 and redoxin to hydroxylate linalool at <10% of the native system turnover; the activity observed for LIN P-450 supported by the CAM redox proteins attains <5% of the native turnover. These observations indicate that the LIN redox proteins cannot support reactions by the CAM components, suggesting as a first hypothesis that the redox potentials of the LIN reductase and redoxin may be greater than those of the corresponding CAM proteins.

Absorption Spectra—The extinction coefficients at various wavelength maxima are reported for oxidized LIN reductase and LIN redoxin in Tables IV and V. The reductase spectrum is typical of a flavoprotein in showing maxima at 275, 381, 456, and 480 nm; the extinction coefficients were essentially identical to those of CAM reductase. The iron sulfide center of LIN redoxin, like the CAM redoxin, shows maxima at 324, 412, and 445 nm with similar extinction coefficients, whereas in the UV at 270 nm the aromatic absorption is markedly less (Fig. 1, Table VII). The extinction coefficients for LIN P-450 in oxidized and reduced states with various ligands are reported in Table VI. The heme content of the P-450 holoenzyme was determined by the pyridine hemochrome measurement of iron protoporphyrin IX (49). In the oxidized substrate-free state, LIN P-450 shows a Soret maximum at 417 nm, characteristic of low spin ferric heme. Upon addition of substrate, the spin equilibrium is shifted in favor of the high spin form, with a Soret maximum at 392 nm. The ferrous-CO complex of LIN P-450 has a maximum at 447 nm with extinction coefficient equal to that for CAM P-450.

LIN Monooxygenase Reaction Products—Substrate oxidation was monitored by gas chromatography of sample extracts taken at intervals from a reconstitution of LIN monooxygenase with NADH and linalool. Two products were detected, with product 1 (P1) appearing as early as 1 min into the reaction and product 2 detected after ~6 min. In order to identify these products, a large-scale reaction mixture was prepared as described under "Materials and Methods." The two products, purified by column chromatography, were then characterized by ^1H NMR, IR, and mass spectra. Spectral data and assignments are reported under "Materials and Methods."

The first product was identified as 8-hydroxylinalool on the basis of the mass spectral molecular ion, m/z = 170 (linalool + 0); IR data supporting –OH as the only oxygen function; ^1H NMR consistent with $-CH_2OH$ at C8 or C9 and nuclear Overhauser enhancement refinement to assign the CH_2OH as *cis* to C6–H, *i.e.* 8-hydroxylation. The second product was identified as 8-oxolinalool on the basis of the mass spectral molecular ion, m/z = 168 (^2H oxidation of P1), IR support for both carbonyl oxygen and aldehydic proton, and NMR analysis supporting the aldehyde assignment *cis* to C6–H. The identifications of P1 and P2 were further supported by comparison with synthetic compounds. Thus, LIN monooxygenase catalyzes two sequential oxidations of linalool, producing first 8-hydroxylinalool and further oxidizing this product to 8-oxolinalool. The oxidation of the alcohol to the aldehyde in the LIN system parallels the conversion of 5-*exo*-hydroxycamphor to 5-ketocamphor by the CAM monooxygenase (Fig. 2). In both systems, each oxidation requires the three protein components, NADH, and O_2. Both reactions were fully inhibited by CO; the preparations were free of detectable alcohol dehydrogenase activity. The occurrence of P-450-catalyzed alcohol oxidation is interesting, since both strains have substantial alcohol dehydrogenase activities for these substrates, with the corresponding enzymes having been purified to homogeneity.[3] It is probable that a second hydroxylation by the P-450 gives rise to transient gem diol adducts that spontaneously dehydrate to the more stable carbonyl compounds. Purification of a P-450-dependent linalool 8-hydroxylase from *Pseudomonas incognita*, strain PpG777, contrasts with the reports by Bhattacharyya *et al.* (6, 50, 51) of a series of

[3] A. J. H. Ullah and I. C. Gunsalus, unpublished results.

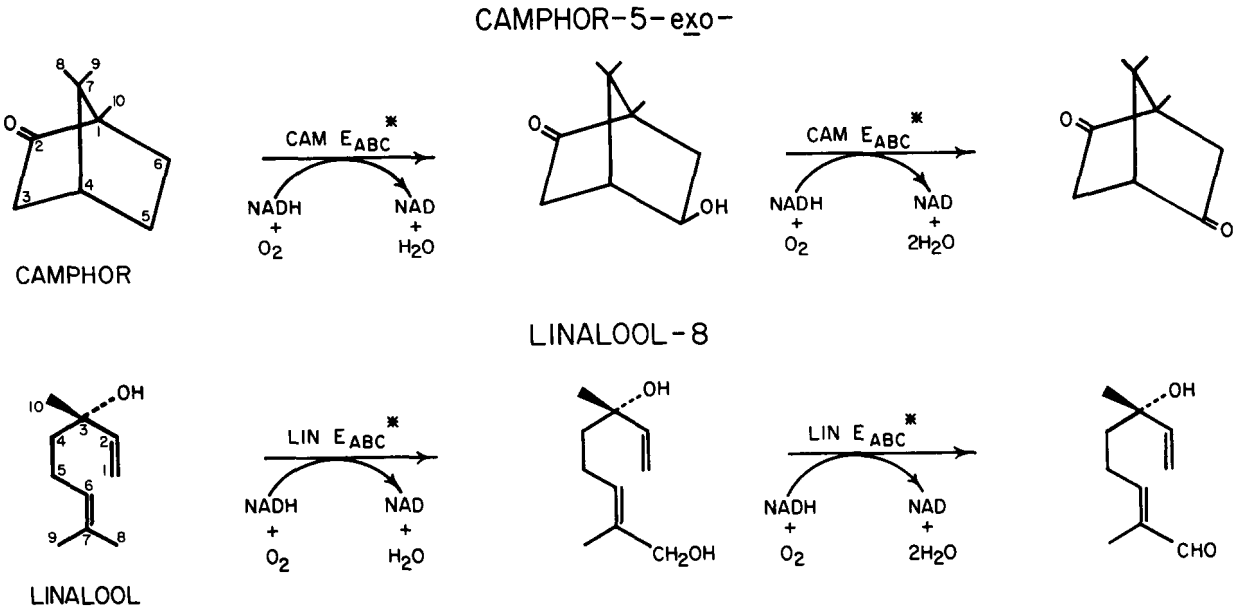

FIG. 2. **Reactions catalyzed by the P-450$_{cam}$ and P-450$_{lin}$ terpene monooxygenases.** *E_{ABC} = FAD reductase, $Fe_2S_2Cys_4$ redoxin, heme thiolate (P-450) monooxygenase.

products indicating also a 10-hydroxylation of linalool from the freshly isolated *P. incognita* culture. The 10-methyl hydroxylation was not observed in the *Psuedomonas* Hardy isolate. The elucidation of the very interesting genetic control of these strains is continuing (55).

The initial characterization of the LIN and CAM monooxygenases indicates that despite the chemical and physical similarities, the component interactions clearly define two unique systems. Spectral characteristics also delineate active site differences in the P-450s substrate binding domains and in the water structure associated with the sixth ligand position (54–57). Current studies focus on the primary and tertiary structure and functional aspects of these two P-450 systems and on the control of the protein expression.

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Supplemental Material to "Protein Components of a P450 Linalool-8-methyl Hydroxylase"

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Materials and Methods

BACTERIAL CULTURE

Pseudomonas incognita strain PpG777 (work #PH651), a P. putida variant, selected from subcultures of the Madyastha isolate (6) by P. Harder, was maintained on minimal phosphate-ammonium-inorganic salts (PAS) agar plates and fed by vapor from 0.1 ml of neat (±)-linalool spread in the petri dish top. Stock cultures were transferred monthly and were stored at 4°C.

The basal PAS medium for liquid culture was prepared from a phosphate-ammonium solution (25 mM KH_2PO_4, 50 mM K_2HPO_4, 150 mM NH_4Cl) supplemented with inorganic salts: 100x salts I containing (g/l): $MgSO_4$, 9.75; $MnSO_4 \cdot H_2O$, 5.0; $FeSO_4 \cdot 7H_2O$, 5.0; $CaCl_2 \cdot 2H_2O$, 0.3; and ascorbic acid, 1.0; 1000x salts II, containing (g/l): $(NH_4)_6Mo_7O_{24}$, 0.185; $CuSO_4 \cdot 5H_2O$, 3.92; $Co(NO_3)_2 \cdot 10H_2O$, 2.5; $ZnSO_4 \cdot 7H_2O$, 109.5; 100x $FeSO_4$/Ascorbate containing (g/l): $FeSO_4 \cdot 7H_2O$, 5.0; ascorbic acid, 1.0. The medium was also supplemented with Bacto yeast extract (Difco) and FMN (Sigma) to final concentrations of 0.1% (w/v) and 2 uM, respectively. The L-broth contained (g/l): Bactotryptone (Difco), 10.0; Bacto yeast extract (Difco), 5.0; and glucose, 1.0; and was supplemented with Cb (100 ug/ml) immediately prior to innoculation.

Tube and flask cultures were incubated at 30°C in a New Brunswick shaker for the specified times. Liquid cultures were started by loop innoculation from the stock plate into 5 ml L-broth. The cultures were incubated 8 hours and transferred at 4% innoculum to 24 tubes containing 5 ml L-broth. After 8 h incubation, four tubes were used as innoculum (4%) for each of six 2-liter Erlenmeyer flasks containing 500 ml basal PAS medium. Growth was monitored turbidometrically at 660 nm with a Cary 219 spectrometer, using a 3 nm spectral band width. When the flask cultures reached A_{660} = 0.4, growth on linalool was initiated by addition of 200 ul/flask (±)-linalool neat (concentration of 2.25 mM). After ~5 h the cultures were actively growing on linalool with A_{660} > 1.0. A second feeding of linalool (267 ul/flask) was made and incubation continued until A_{660} > 2.0. The contents of the six flasks were then used as innoculum for the 28 liter New Brunswick fermentor.

Fermentor cultures were monitored for pH and dissolved oxygen with the appropriate electrodes. The pH was maintained between 6.6 and 6.8 by addition of 3N NaOH as needed. The fraction of dissolved oxygen was calibrated prior to innoculation and maintained at > 40% saturation by increasing the rates of aeration and agitation as necessary. Although it serves as the sole carbon and energy source for the culture, linalool in concentrations > 5 mM was found to be toxic to the cells. Additions of neat linalool were made to produce final concentrations of 1.5 mM through the early exponential phase of growth, and increased to 3.0 mM as the culture density increased. When a rapid rise in dissolved O_2 signaled linalool depletion, the next addition was made.

The 28 l New Brunswick fermentor was charged with 22 l of PAS medium and innoculated with the contents of the six shake flasks. Growth was continued until A_{660} > 4.0. The 25 l culture was then used as innoculum for the New Brunswick 250 l fermentor, previously charged with 175 l of PAS medium. Growth was continued to A_{660} > 8.0 with the fraction of dissolved oxygen > 40%. The oxygen fraction was allowed to decrease to ~10%, during the final phase of growth, to maximize prodution of $P450_{lin}$. When A_{660} > 14.0 and the concentration of $P450_{lin}$, in the culture, reached 1.0 uM, as indicated by the ferrous-CO difference spectrum, the cells were harvested and stored at -20°C as described in reference 28. The dissolved oxygen fraction was monitored throughout the harvest and linalool was added as needed.

PROTEIN PURIFICATION PROCEDURES

Buffers. Two stock buffers were used: 50 mM Tris-Cl, pH 7.4 at 25°C, termed buffer T; and 20 mM potassium phosphate, pH 7.0 (buffer P). The ionic strengths of the buffers were increased for elution of ion exchange columns by addition of KCl; mM KCl concentrations in buffer T are indicated as T-20, T-50,... T-600. A similar nomenclature is used for the phosphate buffers. For the purification of $P450_{lin}$ all buffers contained 175 uM linalool. Buffers used in the purification of LIN-redoxin were supplemented with a 2-mercaptoethanol at 10 mM, while those used for the purification of LIN-reductase were made 5 or 10% in glycerol as noted in the text.

Assays. Analytical measurements followed the procedures of Gunsalus and Wagner (28) with the following modifications. Total protein was estimated with Coomassie Brilliant Blue G-250 according to Sedmark and Grossberg (29) with an ovalbumin standard. After partial purification, protein concentrations were estimated from the 280 nm absorption with correction for the 280/260 absorption ratio. The extinction coefficients, E, mM, for the chromophores were estimated from the corresponding CAM analogs (28): LIN-reductase, E_{456} = 10; LIN-redoxin, E_{328} = 15.6; and $P450_{lin}$ (ferrous CO-ferrous), $E_{446-490}$ = 120. The total system hydroxylase activity was estimated for each component from the substrate-stimulated NADH oxidase activity in the presence of an excess of the other two proteins (0.5 uM P450, 0.5 uM reductase, 25 uM redoxin; limiting component, 50 nM). The unit of activity is defined as 1 nmol NADH oxidized per second.

Purification Protocols

The modifications of the Gunsalus and Wagner procedure (28) reported here, provide a simplified method with improved yields and are equally well suited for the separation and purification of the CAM and LIN components. The procedure for purifying the three components are essentially the same through the preparation of the cell-free extract and the first ion exchange chromatography. The single exception is that for purification of the reductase, all buffers contain 10% glycerol (v/v), and for purification of the P450 and redoxin, buffers contain 175 uM linalool and 10 mM 2-mercaptoethanol, respectively.

Step 1: Cell Free Extract. Storage of cells at -20°C for a minimum of one week before thawing at room temperature is considered to aid in the rupture of the cells and release of protein. A 3 kg portion of cell paste was broken into fine pieces and added to 4/3 volume of room temperature buffer T containing phenylmethylsulfonyl flouride (1 mM). As the cell mass thawed the temperature of the suspension dropped rapidly to 3°C. Manipulation with a spatula gave a homogenous solution. The mixture was then stirred at 5°C and 1.5 g of lysozyme was added. The solution was made 10 mM in $MgCl_2$ and DNAse I and RNAse A were added to final concentrations of 10 ug/ml each. After stirring for 2 h the solution had passed through a viscous phase and returned to a homogenous, non-viscous suspension. The cell debris was removed by centrifugation in a GSA rotor (Sorvall) at 9000 rpm (11,000 x g). The supernatant was decanted and the cell debris resuspended in 1/2 volume of buffer T (5°C); on recentrifugation the yield was 15-20% additional P450, as indicated by ferrous CO assay -- presumed to come from dilution of the aqueous phase of the pellet. The clarified extracts were pooled and adjusted to pH 8.0 by addition of a saturated solution of Tris (free base). The pool was then subjected to DEAE ion exchange chromatography.

Step 2: DE-52 Ion Exchange Chromatography. The first ion exchange chromatography uses two columns in sequence to allow rapid elution of the labile redoxin fraction (column A: 15 cm diameter x 5 cm length; column B: 15 cm diameter x 15 cm length). Column A was equilibrated to buffer T and the extract pool was applied at a flow rate of 1.2 l/h, passing first through a column (9cm x 5cm) packed with CF-11 cellulose fibers (Whatman) to remove any particulate matter. After loading, column A was washed with 600 ml of buffer T-100 and the effluent was discarded. Subsequent effluent from column A was applied to column B, which was previously equilibrated with buffer T-50. As elution was continued with buffer T-100 at the same flow rate, the red-brown P450 band separated from the trailing yellow flavin and brown redoxin bands. After the P450 and reducatse had passed into column B, column A was disconnected and eluted with a 9.6 liter linear gradient of buffers T-100 to T-600. Column B was eluted with an identical gradient. Assay of fractions from column B showed that 70% of the Lin redoxin reductase activity had been coeluted from column A in the P450 band; peak reductase activity coincided with $P450_{lin}$ with elution from column B occurring at 180 mM KCl. The gradient elution of column A eluted the balance of the reductase activity (30%) ahead of the redoxin that eluted at 350 mM KCl. Fractions were assayed and pooled and purification continued according to the individual protocols that follow.

LIN Redoxin Reductase: From both columns, fractions containing the reductase activity were pooled and subjected to ammonium sulfate fractionation.

Step 3: Ammonium Sulfate Fractionation. Over a 30 min period at 0°C, solid ammonium sulfate (enzyme grade, Schwarz-Mann) was added to the pooled fractions from the first DE-52 chromatography to a level of 200 g $liter^{-1}$ (36% of saturation). After stirring for an additional hour at 0°C, the resulting precipitate was removed by centrifugation and discarded. Ammonium sulfate, 200 g $liter^{-1}$, was added to give ~65% of saturation. The precipitate was recovered by centrifugation and suspended in buffer T containing 5% glycerol (v/v), to a final volume of ~300 ml. Complete dissolution was achieved by rapid dialysis under an argon atmosphere against the same buffer.

Step 4: DE-52 Ion Exchange Chromatography II. All buffers used in steps 4-8 of the LIN reductase purification contained 5% glycerol (v/v). The dialyzed enzyme fraction was clarified by centrifugation and applied to a DE-52 column (7.1 x 27.6 cm) equilibrated with buffer T. The column was developed with a linear gradient (1.6 liters) of buffers T-100 to T-300 at a flow rate of 200 ml h^{-1}. Under these conditions, LIN P450 eluted slightly ahead of LIN redoxin reductase activity. The LIN redoxin reductase fractions were combined and the proteins precipitated with $(NH_4)_2SO_4$ (65% saturation) according to the procedure described in Step 3. The precipitate was suspended in buffer T to a final volume of 120 ml with complete dissolution achieved by rapid dialysis under an argon atmosphere.

Step 5: Gel Filtration Chromatography. The dialyzed enzyme fraction from Step 4 was applied to a BioGel P100 gel filtration column (8.8 x 100cm) equilibrated with buffer T. The protein was followed by 200 ml of buffer T containing 20% sucrose (w/v), and the column was developed in the ascending mode with the equilibration buffer at 200 ml h^{-1}. Under these conditions, the LIN redoxin reductase coelutes with LIN P450 fractions; the reductase fractions were combined for the next step.

Step 6: DE-52 Ion Exchange Chromatography III. The combined LIN redoxin reductase fractions from Step 5 were applied to a DE-52 cellulose anion exchange column (4.3 x 40 cm) equilibrated with buffer T. The column was eluted with a step gradient at a flow rate of 120 ml h^{-1}: 1.0 liter of buffer T-90, followed by 2.0 liters of buffer T-105. The LIN P450 was eluted ahead of the LIN redoxin reductase activity. The LIN redoxin reductase fractions were combined and concentrated to 40 ml by ultrafiltration using a PM-10 Diaflo membrane (Amicon, Lexington, Mass.), and then equilibrated to buffer T by rapid dialysis.

Step 7: Affi-Gel Blue Affinity Chromatography. The dialyzed enzyme fraction, from the previous step, was applied to an Affi-Gel Blue (Bio-Rad) column (3.3 x 22.0 cm), equilibrated with buffer T. The column was developed with the equilibration buffer at a flow rate of 60 ml h^{-1}. The remaining LIN P450 was eluted very early, while LIN redoxin reductase activity was slightly retarded by the affinity column and was eluted in the later fractions. The active LIN redoxin reductase fractions were combined and concentrated to 15.0 ml by the ultrafiltration methods described in Step 6.

Step 8: Matrex Gel Blue A Affinity Chromatography. The reductase from the Affi-Gel Blue column (Step 7), was applied to a Matrex Gel Blue A (Amicon) affinity column (1.9 x 38 cm), equilibrated with buffer T. The column was developed with the equilibration buffer at a flow rate of 30 ml h^{-1}. Elution of LIN redoxin reductase was retarded on this affinity column. The active fractions were combined and concentrated to 7 ml by the ultrafiltration methods as described in Step 6, and stored in liquid nitrogen.

LIN Redoxin:

Step 3: Ammonium Sulfate Fractionation. Over a 30 min period at 0°C, solid $(NH_4)_2SO_4$ (enzyme grade, Schwarz-Mann) was added to the redoxin pool from step 2 to a level of 560 g $liter^{-1}$ (85% of saturation). After stirring for an additional 30 min at 0°C, the precipitate was collected by centrifugation. The precipitate was suspended in buffer T-20 to a final volume of ~50 ml and subjected to rapid dialysis under an argon atmosphere to achieve complete dissolution of the proteins.

Step 4: Gel Filtration Chromatography. The dialyzed solution was clarified by centrifugation and applied to a Bio-Gel P-30 gel filtration column (5 x 80 cm), equilibrated with buffer T-20. A 50 ml portion of buffer T-20 containing 20% sucrose was applied before the sample to reduce gravitational effects, and the column was eluted with the equilibration buffer at a rate of 80 ml h^{-1}.

Step 5: DE-52 Ion Exchange Chromatography II. The LIN redoxin pool from Step 4 was applied at a flow rate of 300 ml h^{-1} to a DE-52 cellulose column (6.2 x 19 cm) equilibrated with buffer T. The column was developed with a linear gradient (3.6 liters) of buffers T-150 to T-350 at a flow rate of 150 ml h^{-1}. Fractions were combined and concentrated by ammonium sulfate precipitation of the protein as described in Step 3. The precipitate recovered by centrifugation was suspended in buffer T and dialyzed under an argon atmosphere to achieve complete dissolution and eliminate ammonium sulfate. The dialyzed redoxin pool was made 5 mM in dithiothreitol prior to storage in liquid nitrogen.

LIN P450

Step 1: Ammonium Sulfate Fractionation. The P450 pool from column B (Step 2) was made 1 mM in linalool and fractionated with ammonium sulfate by adding 200 g $liter^{-1}$ (36% saturation) of finely meshed $(NH_4)_2SO_4$ (enzyme grade, Schwarz-Mann) over a 2 h period at 0°C. The precipitate recovered by centrifugation was discarded and a second fraction was obtained from the supernatant by further addition of $(NH_4)_2SO_4$ (140 g $liter^{-1}$, ~60% saturation). The precipitate was suspended to a final volume of ~100 ml in buffer P-20. Complete dissolution was achieved by rapid dialysis under an argon atmosphere against the same buffer.

Step 4: Gel Filtration Chromatography. The dialyzed P450 fraction was applied to a Bio-Gel P-100 gel filtration column (8.8 x 100 cm) equilibrated with buffer P-20. The protein fraction was followed by 200 ml of buffer P-20 containing 20% (w/v) sucrose. The column was then developed in the ascending mode with the equilibration buffer at 200 ml h^{-1}.

Step 5: DE-52 Ion Exchange Chromatography II. The P450 pool from Step 4 was applied at a flow rate of 300 ml h^{-1} to a DE-52 cellulose column (7.0x 28 cm) equilibrated with buffer P. The column was developed with a linear gradient (4.1 liters) of buffers P-50 to P-200 at a flow rate of 170 ml h^{-1}. The P450 peak was eluted at 180 mM KCl. P450 fractions were combined, made 1 mM in linalool and concentrated by ammonium sulfate precipitation (400 g $liter^{-1}$, 65% saturation), according to the procedure described in Step 3. The precipitate was suspended in 50 mM potassium phosphate buffer containing 175 uM linalool. Complete solution was achieved by rapid dialysis under an argon atmosphere against the same buffer. Stock aliquots at ~500 uM were stored in liquid nitrogen.

ANALYTICAL METHODS

SDS polyacrylamide gel electrophoresis was according to Laemmli (30) with 8% total acrylamide mononer, and isoelectric focusing used polyacrylamide gels (5 x 100 mm) containing 2.2% (v/v) ampholine (pH 3.5-5.0) according to Catterall (31), with ovalbumin as standard. Immunodiffusion assays used the methods of Davis et al. (32). Antisera were raised to purified P450 and reductase (CAM and LIN) in female New Zealand rabbits (32) and were purified according to standard procedures (33).

Amino Acid Analyses - The amino acid compositions of the LIN proteins were determined on a Beckman 119CL analyzer at the University of Illinois, Urbana facility. Supplementary data was provided by Dr. G.E. Tarr (34,35) of the University or Michigan Protein Sequencing Facility, and by Dr. K. T. Yasunobu (21,22) of the University of Hawaii, Monoa campus.

All amino acid analyses were performed on the apoproteins. Flavin and iron-sulfide prosthetic groups were removed after precipitation of the apoprotein at 0°C by addition of 20% (w/v) trichloroacetic acid and standing for 0.5 h. Heme was removed from $P450_{lin}$ by the acid butanone method used for $P450_{cam}$ (36). The P450 apoprotein was then precipitated with 20% trichloroacetic acid. The apoproteins were washed twice with diethyl ether/acetone (1:1), dried at ambient temperature, and hyrolyzed for 24 h in vacuo, with 4 N methane sulfonic acid at 115°C. Cysteine content was estimated as cysteic acid in samples oxidized with performic acid (37) prior to hydrolysis.

Amino acid mole fractions were averaged from duplicate measurements. The nearest integer values were calculated from the composition estimated by averaging least squares refinement (38) and an integer composition (plus prosthetic group) in best agreement with the molecular weight estimated from gel filtration data.

Linalool Monoxygenase Reaction Products: Analytical separation and quantitation of reaction products in CH_2Cl_2 extracts were based on GC methods used for metabolites in the camphor system (39). Compounds were separated on columns (1/8 inch x 6 feet, 80/100 Supelcoport as support) with 3% OV-17 (140°C) or 10% carbowax 20M (180°C) as stationary phase. Structures of products from the LIN-monoxygenase were determined using samples purified from a scaled-up reconstitution assay. A reaction volume of 160 ml contained 50 mM potassium phosphate (pH 7.0), 2.2uM each of LIN-P450 and LIN-reductase, 90uM LIN-redoxin, 4.1 mM (±)-linalool, 0.9 mM NADH, and 0.5mM DTT. Glucose-6-phosphate dehydrogenase (10 U) and glucose-6-phosphate (7.2 mM) were added as an NADH regenerating system. The reaction mixture was incubated for 3h at 25°C, under a dioxygen atmosphere (20 psi), after which it was repeatedly extracted with CH_2Cl_2, yielding a mixture of two products. Following solvent evaporation, the residue was chromatographed on a silica gel column (grade 0.5-2mm, Brinkmann). Elution with a step gradient of ethyl acetate/chloroform gave product 2 (15% ethyl acetate) and product 1 (40% ethyl acetate) in pure form. Structural characterizations were based, in part, on spectral assignments.

Product Structure Determination: The first product was identified as 8-hydroxylinalool, based on the following spectroscopic data. ^1H NMR: 1.28 (s, 3H, C10-CH_3), 1.64 (s, 3H, C9-CH_3), 3.95 (s, 2H, -CH_2-OH), 5.03-5.07 (d, 1H, vinylic C1-H), 5.16-5.24 (d, 1H, vinylic C1-H), 5.40 (bt, 1H, C6-H), 5.84-5.97 (dd, 1H, C2-H). NOE data between the C6-H and either the -CH_2OH (21% effect) or the CH_3 (14% effect) at 8 and 9 indicated the hydroxymethyl group is cis to the C6-H. IR: 1640 cm^{-1} (C=C), 3360 cm^{-1} (-OH). MS (70 eV) m/z (rel. intens.): 152 (3, M^+-18), 137 (7), 71 (64), 67 (40), 43 (100).

The second product was identified as 8-oxolinalool from the following data. 1H NMR: 1.34 (s, 3H, C10-CH_3), 1.74 (s, 3H, C9-CH_3), 5.09-5.29 (m, 2H, vinylic C1-CH_2), 5.86-5.99 (dd, 1H, vinylic C2-CH), 6.51 (bt, 1H, C6-H),9.38 (s, 1H, -CHO). Application of Pascual's equation (41) provides a calculated chemical shift of the C6-H, relative to the -CHO group, of 6.40 for the cis isomer and 6.67 for the trans isomer. The observed chemical shift value of 6.51 is more consistent with the cis C8HO isomer than a trans C9HO isomer. This assignment is also in agreement with the structure deduced for P1. IR: 1640 cm^{-1} (C=C), 1685 cm^{-1} (C=O, aldehyde), 2720 cm^{-1} (-CH stretch, aldehyde). MS (70 eV) m/z (rel. intens.) 150 (2, M^+-18), 135(2), 83 (19), 71 (94), 43 (100).

Both products were found to be identical by TLC, GLC, ^1H-NMR, IR and MS data with synthetic compounds. Independent syntheses of authentic P1 and P2 samples, which started from 8-oxolinalyl acetate, will be presented elsewhere.

Instrumentation: Protein optical spectra were recorded at 25°C on a Cary 219 spectrophotometer operating with a 1 nm spectral band width. EPR spectra were measured on a Brucker 9 GHz spectrometer (ER 200D) equipped with an Oxford cryostat and helium transfer system. Magnetic fields were calibrated from the six Mn^{2+} hyperfine lines in SrO powder (42-44). Protein EPR spectra of frozen solutions (1 mM) were recorded with 5-10 G modulation amplitude and 100 kHz modulation frequency at non-saturating microwave power levels (45-47). Computational analyses, including redoxin optical spectra, hand-digitized in 1 nm increments, and amino acid composition data, were processed on a Horizon Northstar computer interfaced (8-bit) to an X-Y plotter. Proton NMR spectra were recorded on a Varian 220 MHz FT-spectrometer (HR220) and Nuclear Overhauser Effect (NOE) experiments (48) were carried out on a Nicolet 360 MHz FT-spectrometer (NT360). Positive chemical shifts (ppm) were downfield from tetramethylsilane (TMS). NMR samples were prepared in $CDCl_3$ as 5% (w/v) solutions that were carefully degassed prior to measurement. Infrared spectra were obtained for neat liquids on a Nicolet 7199 FT-spectrometer. Mass spectra were determined on a Varian MATCH-5. Gas chromatography was performed on a Hewlett-Packard series 5700A Research Gas Chromatograph, equipped with a flame ionization detector.