Properties of the System for the Mixed Function Oxidation of Kaurene and Kaurene Derivatives in Microsomes of the Immature Seed of *Marah macrocarpus*

ELECTRON TRANSFER COMPONENTS[1]

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ABSTRACT

Cytochrome P-450 and cytochrome b_5 at levels of approximately 0.10 and 0.60 nanomole per milligram of microsomal protein were detected by spectral measurements in microsomes prepared from endosperm tissue of immature *Marah macrocarpus* seeds. TPNH-cytochrome *c* reductase, DPNH-cytochrome *c* reductase, andDPNH-cytochrome b_5 reductase activities were also present in these microsomes at levels of approximately 0.060, 0.22, and 0.52 unit per milligram of microsomal protein, respectively. (One unit of reductase is the amount of enzyme catalyzing the reduction of 1 micromole of electron acceptor per minute.) Treatments of microsomes with steapsin or trypsin were not effective in solubilizing any of these electron transport components in detectable form. However, treatment of a microsomal suspension in 25% glycerol with 1% sodium deoxycholate led to the release of about 60% of the protein and each of the above hemoproteins and electron transfer activities to the fraction which was not pelleted after centrifugation for 2 hours at 105,000g. Some *ent*-kaur-16-ene oxidase activity could be detected in the solubilized fraction after removal of the detergent. Cytochrome b_5 and DPNH-cytochrome b_5 reductase activity were largely separated from one another and from an overlapping mixture of TPNH-cytochrome *c* reductase and DPNH-cytochrome *c* reductase when the sodium deoxycholate-solubilized fraction was chromatographed on a DEAE-cellulose column. No cytochrome P-450 or cytochrome P-420 was detected in the column fractions and no *ent*-kaur-16-ene oxidase activity was detected when the column fractions were tested singly or in combination.

The possible participation of these components in the mixed function oxidation of *ent*-kaur-16-ene and a number of its oxidized derivatives catalyzed by these microsomes is discussed in relation to the model which has been developed to explain the function of analogous components in mixed function oxidase reactions in mammalian liver microsomes.

In recent years there has been extensive experimental interest in Cyt P-450-dependent external mixed function oxidase reactions, especially in relation to biosynthetic hydroxylations and drug metabolism. The most thorough investigations have been conducted with mammalian microsomal and mitochondrial systems and with a soluble system from the bacterium *Pseudomonas putida*. (See a monograph edited by Hayaishi [10] and a review

by Gunsalus *et al.* [7] for recent summaries of information about these and other systems.)

Cyt P-450-dependent oxidases have also been reported in higher plant systems, but the characteristics of these have not been very thoroughly described. Murphy and West (22) presented evidence for the involvement of Cyt P-450 in the oxidations of *ent*-kaur-16-ene[2] and some of its oxidized derivatives catalyzed by a microsomal preparation from the endosperm of immature *Marah macrocarpus*[3] seeds. Several other reports have indicated the presence of a CO-binding pigment with spectral and other properties resembling Cyt P-450 in microsomal preparations from etiolated *Pisum sativum* seedlings and cotyledons of *Phaseolus vulgaris* and *Zea mays* (19), from *Arum* spadices (34), and from potato tubers (4). However, no function was ascribed to the P-450 from these sources. The characteristics of the *trans*-cinnamic acid 4-hydroxylase activity in microsomes of pea seedlings (28) and Jerusalem artichoke tubers (2) implicated Cyt P-450 in this oxidation. A recent paper by Potts *et al.* (27) provides definitive evidence for Cyt P450 participation in this 4-hydroxylation of *trans*-cinnamic acid by microsomes from sorghum seedlings and further describes a number of characteristics of the electron transfer reactions associated with the reaction. Finally, Madyastha *et al.* (17) and Meehan and Coscia (21) have shown a role for Cyt P-450 in the 10-hydroxylation of geraniol and nerol by vacuolar preparations from *Vinca rosea* seedlings.

The preceding paper by Hasson and West (9) dealt with the pyridine nucleotide and flavin nucleotide cofactor requirements for the mixed function oxidation of kaurene[4] and some of its oxidized derivatives (kaurenol, kaurenal, and kaurenoic acid) in microsomes from the endosperm of immature *M. macrocarpus* seed. This paper is concerned with the description of some of the electron transfer components found in these microsomal preparations and their possible involvement in these oxidase reactions.

MATERIALS AND METHODS

Microsomal Preparations. The microsomal fraction was prepared from a homogenate of endosperm of immature wild cu-

[2] See Figure 1 of Hasson and West (9) for the structure of this compound.

[3] *Marah macrocarpus* (Greene) Greene was earlier classified as *Echinocystis macrocarpa* Greene. The latter name has been used in earlier publications from our laboratory dealing with the biochemical properties of immature seed of this plant.

[4] Abbreviations: kaurene: *ent*-kaur-16-ene; kaurenol: *ent*-kaur-16-en-19-ol; kaurenal: *ent*-kaur-16-en-19-al; kaurenoic acid: *ent*-kaur-16-en-19-oic acid; 7β-hydroxykaurenoic acid: *ent*-kaur-16-en-7α-ol-19-oic acid or (−)-kaur-16-en-7β-ol-19-oic acid.

cumber (*M. macrocarpus*) seeds as described previously by Hasson and West (8). The microsomal pellets were routinely resuspended at the level of 1 to 3 mg microsomal protein/ml 0.1 M tris-maleate, pH 7.3, which contained 25% glycerol (by volume) under an atmosphere of N_2.

Kaurene Oxidase Activity. Kaurene oxidase was measured as previously described (8). In a typical assay [¹⁴C]kaurene (10,000 cpm; 0.3 nmol), TPNH (0.5–1.0 μmol), and FAD (0.05 μmol) were incubated with the enzyme in a total volume of 1 ml for 45 min at 30 C in a water bath with shaking. The products were extracted into acetone-benzene and the amounts of radioactivity associated with kaurene, kaurenol, kaurenal, kaurenoic acid, and 7β-hydroxykaurenoic acid were determined after chromatography of the extract on a silica gel thin layer as a basis for determining the relative oxidation values (see (ref. 9)).

TPNH- and DPNH-Cytochrome *c* Reductase Activities. These reductase activities were determined spectrophotometrically by a procedure based on that of Masters *et al*. (20). A reaction mixture contained in a total volume of 1 ml: 0.10 μmol either DPNH or TPNH, 0.05 μmol Cyt *c*, 50 μmol tris-maleate buffer, pH 7.3, and the enzyme source to be tested. Reaction was initiated by the addition of the reduced pyridine nucleotide and the change of absorbance at 550 nm was monitored in a Gilford recording spectrophotometer. The rate of Cyt *c* reduction was found to be linear over a period of at least 5 min and was proportional to the amounts of enzyme sample added in the concentration range in which activities were measured. One unit of reductase activity is the amount of enzyme catalyzing the reduction of 1 μmol Cyt *c*/min under these conditions.

DPNH-Cytochrome *b*₅ Reductase Activity. This activity was assayed according to Strittmatter (31) with potassium ferricyanide as the artifical electron acceptor. The incubation mixture contained in a volume of 1 ml: 0.10 μmol DPNH, 0.2 μmol potassium ferricyanide, 50 μmol tris-maleate, pH 7.3, and the enzyme to be tested. Reaction was initiated by the addition of DPNH and the change of absorbance at 420 nm was monitored in a Gilford recording spectrophotometer. The rate of ferricyanide reduction was found to be linear with time over a period of at least 3 min and was proportional to the amounts of enzyme sample added in the concentration range in which activities were measured. One unit of reductase is that amount of enzyme which will catalyze the reduction of 1 μmol ferricyanide/min under these conditions.

Measurement of Hemoproteins. The concentration of Cyt P-450 was determined by the method of Omura and Sato (25) from the CO-difference spectrum of a sample reduced by the addition of either sodium dithionite or TPNH. The absorbance difference between 450 nm and 490 nm was employed and an extinction coefficient of 91 mM⁻¹ cm⁻¹ was assumed. Cyt *b*₅ was estimated according to the method of Omura and Sato (23) from the absorbance differences between 424 nm and 409 nm in the reduced minus oxidized difference spectrum. An extinction coefficient of 185 mM⁻¹ cm⁻¹ was assumed. Spectra were recorded on either an Aminco Chance DW-2 dual wavelength spectrophotometer or a Cary model 15 recording spectrophotometer.

RESULTS

Electron Transfer Components of Microsomes. A CO-difference spectrum for a suspension of microsomes from *M. macrocarpus* endosperm reduced with sodium dithionite is shown in Figure 1 (curve A). Absorption maxima are evident at 450 nm and 423 nm. The 450 nm peak is believed due to Cyt P-450 while the peak at 423 nm may be attributable in part, if not entirely, to Cyt P-420, a denatured form of Cyt P-450.

The absorbance at 450 nm decreased while that at 423 nm increased in time during the course of spectral measurements. This supports the idea that the 423 nm absorbance is due to Cyt
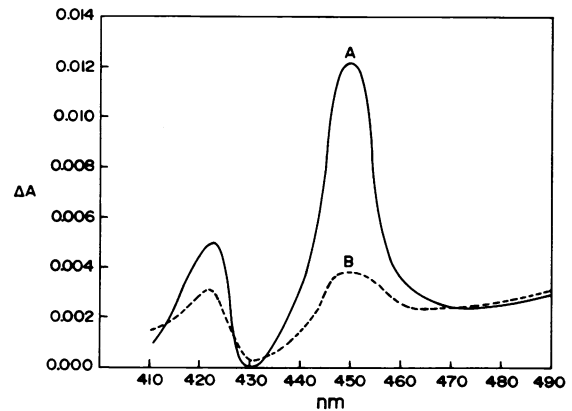

FIG. 1. CO-difference spectra of *M. macrocarpus* microsomal suspensions. Curve A (solid line): microsomal suspensions (1 mg protein/ml) were treated with solid sodium dithionite as the reductant before the addition of CO into one cuvette. The difference spectrum is recorded for a suspension of reduced microsomes containing CO minus an equivalent suspension of reduced microsomes (base-line not shown). The absorbance difference on the ordinate arbitrarily refers to the most negative Δ*A* value in the difference spectrum as zero. Curve B (dashed line): conditions were as for curve A except that 5 × 10⁻⁴ M TPNH replaced sodium dithionite as the reductant.

P-450 and indicates the instability of Cyt P-450 under these conditions. The presence of 0.11 nmol Cyt P-450 and 0.023 nmol Cyt P-420/mg protein can be calculated from the initial spectral measurements made on the suspension shown in Figure 1, curve A. The changes in the spectrum after 30 min standing at ambient temperature indicated that the same suspension contained 0.074 nmol Cyt P-450 and 0.034 nmol Cyt P-420/mg protein. After 60 min of standing the suspension contained only 0.037 nmol Cyt P-450 and 0.052 nmol Cyt P-450/mg protein. The ratio of 450 nm to 423 nm absorbance was increased if the microsomal suspension was prepared in 25% glycerol and maintained under an N_2 atmosphere. Also the 450 nm peak was stable over a longer period of time under these conditions. Similar effects of these conditions on the balance of Cyt P-450 and P-420 in liver microsomes were reported by Sato *et al*. (30). A similar CO-difference spectrum was observed with TPNH as the reductant rather than sodium dithionite (Fig. 1, curve B), but the specific absorbance of both the 450 nm and 423 nm peaks was much less.

The presence of Cyt *b*₅ in the microsomes was indicated by the difference spectrum between a reduced and oxidized microsomal suspension. A prominent peak at 424 nm and a smaller one at 554 nm are typical of Cyt *b*₅ as reported by Omura and Sato (24). DPNH-Cyt *c* reductase, TPNH-Cyt *c* reductase, and DPNH-Cyt *b*₅ reductase activities were detected in microsomal suspensions by spectrophotometric assays as described under "Methods."

Table I summarizes the content of these hemoproteins and the reduced pyridine nucleotide-hemoprotein reductase activities in one preparation of *M. macrocarpus* endosperm microsomes. The results are typical of a series of determinations made on different microsomal preparations.

Solubilization of Microsomal Electron Transfer Components. Several procedures to extract the electron transfer components from the microsomes into the "soluble" fraction which would not pellet after centrifugation at high speed have been tested. Enzymic digestions of microsomes by steapsin and trypsin were attempted as described by Omura and Sato (24) for removal of Cyt P-450 from mammalian liver microsomes. These enzyme treatments failed to release any detectable electron transport components or kaurene oxidase activity to the supernatant fraction after high speed centrifugation. The three reductase activities and Cyt *b*₅ were still detectable in the pellet after treatment

with the digestive enzymes. Most of the CO-binding Cyt was found in the 423 nm form in the pellet after digestion with enzymes, whereas Cyt P-450 was still evident in control microsomes not treated with enzyme. Also the overall kaurene oxidase activity was greatly reduced in the enzyme-treated pellets in comparison with the controls.

Solubilization by treatment with detergents was more successful. The approach utilized for solubilization of the activities and subsequent resolution of the components by ion exchange chromatography were based on those pioneered for hepatic microsomes by Lu and Coon (13). (See also a recent review on the resolution and reconstitution of the liver microsomal enzyme system [14].) The results of treatment with sodium deoxycholate are summarized in Table II. About 60% of the microsomal protein was present in the supernatant fraction after treatment with this detergent. Sodium deoxycholate treatment also released into the supernatant fraction in detectable form 43% of the Cyt b_5, 30% of the TPNH-Cyt c reductase, 49% of the DPNH-Cyt c reductase, and 46% of the DPNH-Cyt b_5 reductase originally present in the microsomes. About 38% of the original Cyt P-450 plus Cyt P-420 was recovered in the supernatant

fraction, but only one-third of this was in the form of P-450, whereas the original microsomal pellet had 70% of the total as P-450. The kaurene oxidase activity was completely inhibited by the levels of detergent used to effect solubilization. However, after the supernatant fraction was at least partially freed of detergent by a combination of Sephadex G-25 column chromatography and concentration of the void volume fraction on a Diaflo XM-50 membrane, it showed 24% of the kaurene oxidation activity initially observed in the microsomes.

Triton X-100 treatment of microsomes under similar conditions also solubilized a reasonable fraction of the microsomal protein (72%). Cyt b_5 (47%), TPNH-Cyt c reductase (60%), and DPNH-Cyt b_5 reductase (76%) were also detected in the supernatant fraction. However, neither Cyt P-450 nor DPNH-Cyt c reductase activity were detected in either the supernatant or pellet fractions after the Triton X-100 treatment. The absence of these two components may account for the fact that no kaurene oxidase activity could be found in either the pellet or supernatant fractions after detergent treatment, even after the fraction had been processed on a Sephadex G-25 column and Diaflo XM-50 membrane as described above.

Table I. *Levels of Electron Transfer Components Detected in M. macrocarpus Endosperm Microsomes and Microsomes from Other Sources*

	M. macrocarpus[a]		Sorghum seedlings[b]	Rat liver-control[c]	Rat liver-phenobarbital treated[c]
	per mg. protein	per 100 ml endosperm	per mg protein	per mg protein	per mg protein
Cytochrome P-450	0.11 [d]	2.4 [d]	0.10[d]	0.24 [d]	1.10 [d]
Cytochrome b_5	0.61 [d]	13.5 [d]	0.55[d]	0.54 [d]	0.48 [d]
TPNH-cyt c reductase	0.060[e]	1.32[e]	–	0.052[e]	0.226[e]
DPNH-cyt c reductase	0.215[e]	4.75[e]	–	1.24 [e]	0.54 [e]
DPNH-cyt b_5 reductase	0.524[e]	11.0 [e]	–	–	–

[a] Determined in this study by the procedures described under "Materials and Methods."

[b] Data from Potts *et al.* (27).

[c] Data from Orrenius *et al.* (26). The results are expressed in different units from those originally presented. Cyt P-450 was converted to nmol mg protein^{-1} from $\Delta E_{450-500} \times$ mg protein^{-1} by assuming an extinction coefficient of 91 mM^{-1} cm^{-1}. Cyt b_5 was converted to nmol $\times$ mg protein^{-1} from $\Delta E_{427-410}$ by assuming an extinction coefficient of 185 mM^{-1} cm^{-1}. The TPNH-cyt c reductase and DPNH-cyt c reductase values were converted from μmol reduced pyridine nucleotide oxidized $\times$ min$^{-1} \times$ mg protein^{-1} to μmol Cyt c reduced $\times$ min$^{-1} \times$ mg protein^{-1} by multiplying by 2.

[d] Nmol.

[e] μmol electron acceptor reduced $\times$ min^{-1}.

Table II. *Localization of Electron Transfer Components after Treatment of Microsomes with Sodium Deoxycholate*

Microsomes (25 mg protein derived from about 250 ml endosperm) were resuspended in 2.5 ml 0.05 M tris-chloride (pH 7.2) containing 25% glycerol and 1% sodium deoxycholate. The suspension was stirred continuously at 4 C under an N_2 atmosphere for 2 hr. After this treatment the suspension was centrifuged at 105,000g for 2 hr. The resulting supernatant and pellet fractions were analyzed for their contents of electron transfer components by the procedures described under "Materials and Methods." The amount of protein taken for assays for kaurene oxidase activity was in all cases 0.1 mg. The levels of the components in intact microsomes are presented for comparison.

		After detergent treatment	
	Microsomes	Pellet	Supernatant
Total protein	32 mg	9 mg	20 mg
Cytochrome P-450	0.073 nmole/mg protein	None detected	0.021 nmole/mg protein
Cytochrome P-420	0.031 nmole/mg protein	0.02 nmole/mg protein	0.042 nmole/mg protein
Cytochrome b_5	0.65 nmole/mg protein	0.13 nmole/mg protein	0.45 nmole/mg protein
TPNH-cytochrome c reductase	0.062 unit/mg protein	0.014 unit/mg protein	0.03 unit/mg protein
DPNH-cytochrome c reductase	0.23 unit/mg protein	0.025 unit/mg protein	0.18 unit/mg protein
DPNH-cytochrome b_5 reductase	0.53 unit/mg protein	0.096 unit/mg protein	0.38 unit/mg protein
Kaurene oxidation activity	23% relative oxidation	16% relative oxidation[a]	9% relative oxidation[a]

[a] After detergent removal with a Sephadex G-25 column. No activity was detected before detergent removal.

are also presented in Table I. It can be seen that much higher concentrations of Cyt P-450 are present in hepatic microsomes, especially after drug induction. Furthermore, 100 g liver yields 400 to 500 mg microsomal protein, whereas 100 ml endosperm (derived from an average of 500 immature seeds) contains only 15 to 20 mg microsomal protein. There are no known means of increasing the concentration of Cyt P-450 or other electron transport components in the plant microsomes comparable to the inductions by drugs in liver. These facts have complicated the task of adequately characterizing the electron transfer components in the plant system and of demonstrating their role in the mixed function oxidase reactions.

Although it has been possible to solubilize and fractionate the electron transfer components, the reconstitution of an active system in a reproducible manner, even in the presence of exogenous phospholipids, has not been achieved. Had this approach worked, it could have provided direct evidence for the participation of the individual components in the overall process. The failure to obtain an active system may well have been due to loss of Cyt P-450 during the manipulations required for solubilization and fractionation. The Cyt P-450 detectable spectrally after application of these procedures was either greatly reduced or absent. Even control microsomal suspensions not treated with detergents, but held at 4 C in 25% glycerol under an N_2 atmosphere for 30 hr, had lost almost all of their capacity for kaurene oxidation. It should be recalled that the initial concentration of Cyt P-450 in these preparations is also rather low. Of course, it is possible that other required components had been inactivated by the detergent treatment as well.

Several properties of the endosperm microsomal oxidations indicate that the electron transfer components are serving in a manner similar to those proposed for analogous components in mammalian microsomes shown in Figure 3. (a) The involvement of Cyt P-450 was deduced in earlier work from the characteristics of inhibition of the oxidase reactions, especially the inhibition by CO and the maximal photo-reversal of this inhibition by 450 nm light (22). (b) Cyt c has been shown to inhibit drug oxidations in liver microsomes (6), presumably by diverting the flow of electrons from TPNH to Cyt P-450 at the level of fp_T and possibly also from DPNH to Cyt P-450 at the level of Cyt b_5. Cyt c had a similar inhibitory effect on kaurene oxidation in the endosperm microsomes. (c) The synergistic stimulation of the rates of kaurene oxidation by added DPNH in the presence of low levels of TPNH reported in the accompanying paper (9) is very reminiscent of the situation in hepatic microsomes (3). This was a major factor in prompting Estabrook et al. to propose the microsomal electron transport model with two reduction steps in P-450-dependent oxidations pictured in Figure 3. (d) The synergistic stimulation of the rates of kaurene oxidation by added FMN in the presence of low levels of FAD reported in the accompanying paper (9) can also be rationalized in terms of this model if it is assumed that both FMN and FAD have a role in the associated reductases as they do in mammalian microsomes.

The DPNH-Cyt c reductase and the TPNH-Cyt c reductase detected in the plant microsomes in the present study are probably different enzymes just as they are in animal microsomes even though they eluted in overlapping positions from the DEAE-cellulose column used for the fractionation of deoxycholate-solubilized microsomes. For example, the TPNH-Cyt c reductase activity was little affected by treatment with Triton X-100, while the DPNH-Cyt c reductase was totally inactivated after exposure to this detergent. One feature of the DPNH-Cyt c reductase activity from the present work does seem anomalous. The mammalian microsomal DPNH-Cyt c reductase require both fp_D and Cyt b_5 for activity. However, the DEAE-cellulose column fractionation of deoxycholate-treated microsomes (Fig. 2) seems to have resolved both Cyt b_5 and DPNH-Cyt b_5 reductase activity (fp_D) from the DPNH-Cyt c reductase. Either the

detergent treatment only partially resolved the components and somehow the DPNH-Cyt c reductase retains some Cyt b_5 and DPNH-Cyt b_5 reductase which are not revealed by assays for those individual components or DPNH-Cyt c reductase is of a different nature in this case.

The report of Potts et al. (27) is the only one dealing substantially with the properties of a Cyt P-450-dependent oxidase system from a higher plant source. With respect to the microsomal system from sorghum seedlings which catalyzes the 4-hydroxylation of trans-cinnamic acid, they report levels of Cyt P-450 and Cyt b_5 very similar to those found in the endosperm microsomes. Their evidence for CO-inhibition and its photoreversal clearly implicate the Cyt P-450 in the hydroxylase reaction. They find that TPNH is utilized more efficiently than DPNH as the reduced pyridine nucleotide electron donor in much the same way as is true of kaurene oxidation in microsomes (9). A stimulatory effect on the hydroxylation rate is seen when 1 mM DPNH and 1 mM TPNH are included together in comparison with either reduced pyridine nucleotide alone under conditions such that the overall rate of hydroxylation is inhibited by the addition of catechol. This result is interpreted as support for the proposal that two electron transfer pathways are operating as proposed by Hildebrandt and Estabrook (11). The effects of a number of inhibitors or potential inhibitors are also generally in accord with the types of electron transfer pathways proposed for the mammalian microsomal systems. Although it is not possible to compare in detail the results of their work with the properties for the M. macrocarpus microsomal oxidases presented in this paper because of the somewhat different experimental approaches made, it seems that these two systems have many features of their electron transport systems in common.

Clearly there is much yet to be learned about the characteristics of Cyt P-450-dependent mixed funtion oxidations in higher plant systems. But at the level at which it has been possible to make comparisons to date, it seems they have many features in common with the hepatic microsomal Cyt P-450-dependent oxidase.

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