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Characterization and Solubilization of Kaurenoic Acid Hydroxylase from *Gibberella fujikuroi*[1]

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A key step in gibberellin biosynthesis is the conversion of *ent*-kaurenoic acid to *ent*-7α-hydroxykaurenoic acid, mediated by the enzyme kaurenoic acid hydroxylase. A cell-free system obtained from *Gibberella fujikuroi* (Saw.) Wr. was used to characterize kaurenoic acid hydroxylase activity. Microsomal preparations from disrupted fungal cells, in the presence of O_2 and NADPH, converted [17-^{14}C]*ent*-kaurenoic acid to oxidation products that were separated by high-performance liquid chromatography and identified as *ent*-7α-hydroxykaurenoic acid and gibberellin A_{14} by combined gas chromatography-mass spectrometry. Flavin adenine dinucleotide and the chloride salts of several monovalent cations stimulated the conversion of *ent*-kaurenoic acid to these products, whereas CO and a number of known inhibitors of cytochrome P-450-dependent reactions, including paclobutrazol, tetcyclacis, BAS 111..W, flurprimidol, triarimol, metyrapone, and 1-phenylimidazole, significantly reduced kaurenoic acid hydroxylase activity. Kaurenoic acid hydroxylase was solubilized from fungal microsomes by treatment with 1 M KCl. The properties of the enzyme noted above suggest that kaurenoic acid hydroxylase from *G. fujikuroi* is a cytochrome P-450-dependent monooxygenase.

Within the last few decades GAs have been recognized as vital to the growth and development of plants. These compounds are involved in diverse processes such as seed germination, stem elongation, flowering, and fruit development (Graebe, 1987). Cell-free extracts obtained from *Gibberella fujikuroi* (Saw.) Wr., a fungal pathogen of rice, have been used to study GA biosynthesis. Although *G. fujikuroi* is not a higher plant, it has a complete set of GA biosynthetic enzymes and produces large quantities of GAs (Bearder, 1983).

GAs are produced by the isoprenoid pathway, with mevalonate as an early intermediate. Kaurene is the first committed compound in GA biosynthesis, which is oxidized through two intermediates to kaurenoic acid. An additional oxidative conversion, mediated by kaurenoic acid hydroxylase, produces 7α-hydroxykaurenoic acid. In the fungus this compound then can be converted through GA_{12}-aldehyde and GA_{12} to GA_{14} (Fig. 1).

The steps in the conversion of kaurene to 7α-hydroxykaurenoic acid are common to the biosynthesis of all of the many GAs. Therefore, knowledge of the enzymes mediating these conversions is important for understanding the regulation of all GA production. The enzymes catalyzing the oxidations from kaurene to kaurenoic acid are microsomal and are Cyt P-450 dependent (Murphy and West, 1969). Cyts P-450 constitute an important group of monooxygenases that is just beginning to be understood in plant systems (for recent reviews of plant Cyt P-450, see Donaldson and Luster, 1991; Durst, 1991; Mihaliak et al., 1993). Our laboratory has made progress on the solubilization and resolution of components involved in the oxidation of kaurene to kaurenoic acid in *G. fujikuroi* (Hazebroek et al., 1989), and more recently, Ashman et al. (1990) reported the further characterization of this enzyme system.

A series of investigations of kaurenoic acid metabolism in cell-free enzyme preparations from *G. fujikuroi* was carried out by Nakata (1972). Microsomes were found to be responsible for the catalytic conversion of kaurenoic acid to a group of more polar metabolites, with 7α-hydroxykaurenoic acid as the initial product. Evidence was also obtained for the further conversion of 7α-hydroxykaurenoic acid to GA_{12}-aldehyde, GA_{12}, and GA_{14}. The enzyme system described in this earlier work had several features characteristic of a Cyt P-450-dependent monooxygenase. The work described in the present report utilized this information as a starting point for the further elucidation of the properties of kaurenoic acid hydroxylase from *G. fujikuroi*.

A significant hindrance to our understanding of the GA biosynthetic pathway is the lack of purified enzymes specifically related to this pathway. The availability of a pure enzyme involved in GA biosynthesis, such as kaurenoic acid hydroxylase, would allow for the production of antibodies against this enzyme, and these could be used for immunolocalization studies (to determine the location of this enzyme in the plant or fungal cell) and for studies of the developmental regulation of GA production. Pure enzyme would also be amenable to protein sequencing, which would lead

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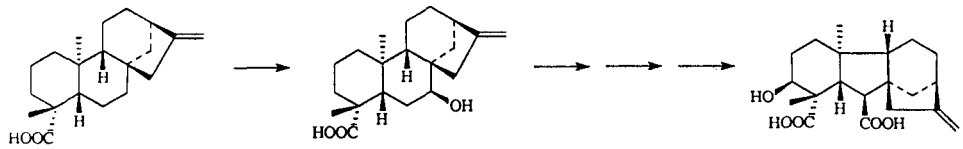
Abbreviations: BAS 111..W, 1-phenoxy-3-(1H-1, 2, 4-triazol-1-yl)-4-hydroxy-5,5-dimethylhexane; FAD, flavin adenine dinucleotide; flurprimidol, [α-(1-methylethyl)-α-[4-(trifluoromethoxy)phenyl]-5-pyrimidine-methanol]; 7α-hydroxykaurenoic acid, *ent*-kaur-16-en-7α-ol-19-oic acid; kaurenal, *ent*-kaur-16-en-19-al; kaurene, *ent*-kaur-16-ene; kaurenoic acid, *ent*-kaur-16-en-19-oic acid; kaurenol, *ent*-kaur-16-en-19-ol; MeOH, methanol; metyrapone, 2-methyl-1,2-di-3-pyridyl-1-propanone; paclobutrazol, 1-(4-chlorophenyl)-4,4-dimethyl-2-(1, 2, 4-triazol-l-yl)pentan-3-ol; tetcyclacis, 5-(4-chlorophenyl)-3,4,5,9,10-pentaazatetracyclo-5,4,$10^{2,6}$,$O^{8,11}$-dodeca-3,9-diene; triarimol, α-(2, 4-dichlorophenyl)-α-phenyl-5-pyrimidine methyl alcohol.



ent-kaurenoic acid *ent*-7α-hydroxykaurenoic acid GA_{14}

Figure 1. GA biosynthetic pathway from *ent*-kaurenoic acid to GA_{14} in *G. fujikuroi*.

to the production of probes to detect mRNA, cDNA, or genomic DNA coding for this and other Cyt P-450 enzymes. An unambiguous protein sequence would be very helpful in designing the primers necessary to isolate the gene for kaurenoic acid hydroxylase and other closely related genes.

With these goals in mind we have characterized and started to purify kaurenoic acid hydroxylase from *G. fujikuroi*. In this paper, we describe properties of the microsomal hydroxylase in cell-free preparations; these properties are consistent with a Cyt P-450 nature for this catalyst. This enzyme was also solubilized from microsomal membranes with 1 M KCl.

MATERIALS AND METHODS

Fungus Culture

The stock culture of *Gibberella fujikuroi* (Saw.) Wr., isolate GF-1a, was obtained from B.O. Phinney and maintained on potato dextrose agar. Blocks of mycelia were used as inoculum for 50-mL liquid cultures in 250-mL Erlenmeyer flasks. The Glc and mineral medium has been previously described (Coolbaugh et al., 1978) except that $ZnSO_4 \cdot 7H_2O$ was substituted for $ZnSO_4 \cdot H_2O$. Cultures were grown for 3 d at 23°C on an orbit shaker, 150 rpm, under room light supplemented with continuous incandescent illumination. After several successive 1-mL transfers of the fungus to 50-mL cultures, 10 mL of suspension were used to initiate 1-L cultures in 2-L flasks. These cultures were grown under the same conditions as described above.

Enzyme Preparation

After 3 d of growth, the mycelia were harvested by vacuum filtration on Whatman No. 1 filter paper. In earlier studies, Johnson (1988) determined that fungal growth begins to slow down after 3 d; yet, GA_3 production is still in the linear phase at this time. The mycelial mats were each rinsed twice with 500 mL of ultrapurified H_2O and twice with 500 mL of 50 mM Tricine (pH 7.7). Cultures were checked for contamination by microscopic examination of the filtrates and by subculturing the fungus and filtrates on potato dextrose agar. Fresh mat weight was typically 25 to 30 g.

Each mat was frozen in liquid N_2 and then disrupted under 20,000 psi in a chilled Sagers press (Sagers, 1962). Crushed mycelia were homogenized in ice-cold 50 mM Tricine/250 mM Suc/10 mM 2-mercaptoethanol (pH 7.7) at a ratio of 4 mL of buffer to 1 g of fresh mycelia weight in a Teflon-to-glass tissue grinder. The supernatant resulting from a 10,000*g* centrifugation of the homogenate for 15 min at 4°C was filtered through glass wool to remove lipid and then ultracentrifuged at 150,000*g* for 90 min at 4°C. This resulted in a microsomal pellet (P_{150}) and a supernatant fraction (S_{150}). The pellet was resuspended in ice-cold homogenization buffer at a ratio of 1 mL of buffer to 1 g of starting mycelia weight.

Preparation of Labeled Substrate

The synthesis of [17-^{14}C]kaurenoic acid began with the natural compound 16α-(−)-kauran-17-monomethyl ester-17,19-dioic acid (provided by P.R. Jefferies). The procedure involves the conversion of the 17-monomethyl ester to the 17α-hydroxy ester based on the methods of Pfeffer and Silbert (1971) and Wasserman and Lipshutz (1975). After saponification of the 17α-hydroxy ester, a chromium trioxide oxidation produced the 17-norketone. Further conversion of the ketone by a Wittig reaction involving [^{14}C-methyl]triphenylmethyl phosphonium bromide yielded [17-^{14}C]*ent*-kaurenoic acid (1.4 mg, specific activity = 0.330 MBq μmol^{-1}). The product was identified by its mass spectrum (model MS-9 high resolution double-focusing sector field mass spectrometer, A.E.I., Ltd.) in comparison with the mass spectrum of an authentic reference standard, also provided by P.R. Jefferies (M^+ 302; m/z 287, 259, 243, 241, 213, 187, 159, and 121).

Enzyme Assays

Oxidation of kaurenoic acid by fungal preparations was assayed in reaction mixtures containing 1.0 μM [^{14}C]kaurenoic acid, 5.0 μM FAD (disodium salt), 50 μM NADPH (tetrasodium salt), and P_{150} protein. Each mixture was brought to 1 mL and pH 7.7 with 50 mM Tricine/250 mM Suc/10 mM 2-mercaptoethanol. Mixtures were incubated in 20-mL glass scintillation vials at 30°C on an orbit shaker, 150 rpm, for 10 min. Reactions were stopped by the addition of 1 mL of acetone. Products were extracted with 1 mL of benzene:acetone (3:1, v/v), followed by two 1-mL ethyl acetate extractions at pH 3. The organic extract was dried under vacuum on a rotary evaporator at 35 to 40°C, and reaction products were transferred to 1.5-mL sample vials for HPLC analysis. Samples were brought to 70% MeOH.

Separation and Identification of Reaction Products

Reaction products were separated by reversed phase HPLC. Samples were loaded onto a 4.6- × 250-mm Ultrasphere (5 μm) C_{18} column (Beckman) and eluted with a water-methanol gradient at 1 mL min^{-1}. Solvents were HPLC grade MeOH and ultrapurified H_2O that was acidified to pH 3 with H_2PO_4. The gradient began at 70% MeOH, then increased to

100% MeOH from 1 to 11 min, and remained at 100% MeOH for 20 min. Elution of radioactive products was monitored by a Beckman 171 radioisotope detector.

Fractions containing enzymic products from several HPLC separations were pooled and dried under vacuum. Samples were methylated by excess ethereal diazomethane. The methylated samples were dried again and redissolved in 5 μL of pyridine and 3 μL of bis-(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane (Regis). Derivatized samples were separated and identified by a Hewlett-Packard 5890 gas chromatograph coupled to a 5970B mass selective detector. Samples were introduced into a 30-m × 0.25-mm (i.d.) fused-silica capillary column with a 0.25-μm dimethyl polysiloxane stationary phase (DB-1; J&W Associates) in a splitless mode. After 1 min at 100°C the column temperature was increased to 280°C at a rate of 10°C min^{-1}. The injection and transfer line temperatures were 300°C, and the column head pressure of the He carrier gas was 20 kPa. The MS electron voltage was set at 70 eV, and full-scan mass spectra were recorded at a rate of 0.68 scan s^{-1}.

Inhibition Studies

Enzyme assays were conducted as described above using 110 μg of P_{150} protein. Solutions of Cyt *c*, paclobutrazol, tetcyclacis, BAS 111..W, flurprimidol, triarimol, metyrapone, 1-phenylimidazole, or piperonyl butoxide were added to the assays. Paclobutrazol was from ICI, tetcyclacis and BAS 111..W from BASF, flurprimidol and triarimol from Eli Lilly, and metyrapone, 1-phenylimidazole, and piperonyl butoxide from Sigma. For the CO studies, reactions with 190 μg of P_{150} protein were in Warburg vessels connected in series to the appropriate gas mixture at a total gas flow rate of 100 mL min^{-1}. Atmospheres were equilibrated over 10 min, and the reaction mixtures were maintained on ice. Reactions were initiated by the addition of the NADPH from a side arm of the vessel and then incubated at 30°C for 10 min.

Solubilization

Fungal microsomes were prepared as described above except that the microsomal pellet (P_{150}) was resuspended in 0.1 M PO_4 (pH 7.7) containing 20% (v/v) glycerol, 10 mM EDTA, 0.25 mM PMSF, 0.10 mM DTT, and protease inhibitors. The protease inhibitors were added to the buffer to give the following final concentrations: 1 mg mL^{-1} each of leupeptin, chymostatin, and pepstatin; 2 mg mL^{-1} of antipain; 5 mg mL^{-1} of benzamidine; and 0.015 to 0.030 trypsin inhibitor unit mL^{-1} of aprotinin (Ronnett et al., 1984). KCl was added to the resuspended P_{150}, and the mixture was rapidly stirred in a glass beaker on ice. After 60 min of stirring, the solubilization mixture was ultracentrifuged at 150,000*g* for 60 min at 4°C to produce a soluble fraction (PS_{150}) and a pellet (PP_{150}).

Protein Determinations

Protein concentrations were determined by the Bio-Rad Standard Protein Assay based on the method of Bradford (1976) with BSA as a standard.

RESULTS

Product Identification

Three radiolabeled compounds were separated by HPLC at average retention times of 9.7, 13.7, and 20.3 min. The material at 20.3 min corresponded with unreacted kaurenoic acid. The peak at 13.7 min cochromatographed with the 7α-hydroxykaurenoic acid standard. This HPLC peak was shown indeed to contain 7α-hydroxykaurenoic acid by GC-MS analysis and comparison with the spectrum of the authentic methyl ester and trimethylsilyl ether of 7α-hydroxykaurenoic acid obtained from *Solidago rigida* (provided by J.P. Hazebroek). The characteristic ions (m/z) and their abundances in the sample were 404 (12), 389 (5), 345 (1), 314 (72), 301 (10), and 255 (68). The HPLC peak centered at 9.7 min was also analyzed by GC-MS and contained GA_{14}. The characteristic ions and their abundances in this sample were 448 (2), 433 (5), 416 (25), 388 (16), 298 (58), and 287 (70), which correspond to the published spectrum of the methyl ester and trimethylsilyl ether of GA_{14} (Crozier and Durley, 1983). No other products of GA biosynthesis were observed in either sample. An unidentified HPLC peak appeared at 16.5 min in reaction samples that contained no fungal protein. This nonenzymic oxidation product of kaurenoic acid was minimal in reactions containing enzyme preparation.

Characterization of Kaurenoic Acid Oxidation

The enzymic oxidation of kaurenoic acid by fungal microsomes had an absolute requirement for NADPH or NADH and was stimulated 2-fold by the addition of FAD (Table I). The experiment shown in the table was repeated with virtually identical results. In experiments in which reaction vials were sealed and the sample atmosphere was flushed with N_2, the hydroxylase activity was reduced by at least 85% (data not shown) in comparison to the air-atmosphere controls, indicating a requirement of O_2 for enzyme function.

Optimal oxidation conditions for 1-mL assays were determined. Using 1.0 μM [^{14}C]kaurenoic acid and a reaction time of 10 min at pH 7.7, we found a protein concentration of up to 300 μg mL^{-1} to be in the linear range of enzyme activity. The presence of monovalent chloride salts (NaCl, KCl, and LiCl) significantly stimulated enzymic conversion (Fig. 2). Each salt yielded an enhancement of at least 50% at a

Table I. *Cofactor requirements for the enzymic oxidation of kaurenoic acid*

Complete enzyme assays contained 1.0 μM [^{14}C]kaurenoic acid, 5.0 μM FAD, 50 μM NADPH, 100 mM NaCl, and 110 μg of P_{150} protein in 50 mM Tricine/250 mM Suc/10 mM 2-mercaptoethanol.

Reaction Mixture	Products (±SE)	Relative Oxidation
	pmol	
Complete	689 ± 10	1.00
Minus P_{150}	0	0
Minus FAD	318 ± 09	0.46
Minus NADPH	0	0
Minus NADPH, plus NADH[a]	129 ± 13	0.19

[a] Fifty micromolar NADH.

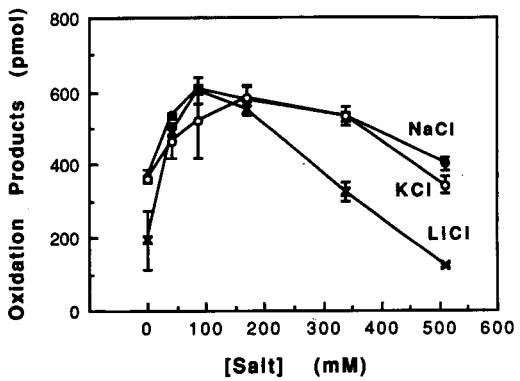


Figure 2. The enhancement of kaurenoic acid hydroxylase activity with the addition of monovalent chloride salts (pmol of products ± SE). Each enzyme assay contained 170 μg of microsomal protein.

concentration of 100 mM in the reaction mixture. On the contrary, 100 mM $CaCl_2$ eliminated all detectable activity. Suc was added to reaction mixtures at the same calculated osmotic potential as 50, 100, and 200 mM NaCl, but no enhancement of activity was seen (data not shown) as compared to the increase seen with the addition of NaCl.

The supernatants resulting from 10,000*g* centrifugation were separated by ultracentrifugation into soluble (S_{150}) and microsomal (P_{150}) fractions. Through two independent experiments it was found that 16% of the total recovered kaurenoic acid hydroxylase activity was in the S_{150}, whereas the majority of the activity (84%) was in the P_{150}. Greater than 50% of the microsomal enzyme activity is lost if the P_{150} is maintained on ice for 1 h. However, P_{150} has been stored at −70°C for several months without any appreciable loss of activity.

Effects of Cyt P-450 Inhibitors

The activity of kaurenoic acid hydroxylase under a 90% CO:10% O_2 atmosphere was reduced to half the activity found under 90% N_2:10% O_2 controls (Table II). An 80% CO:20% O_2 atmosphere also inhibited the hydroxylase activity but to a lesser extent. It is important to note that we have made several unsuccessful attempts to reverse CO inhibition by blue or white light.

The activity of this enzyme system was also measured in the presence of Cyt *c*, paclobutrazol, tetcyclacis, BAS 111..W, flurprimidol, triarimol, metyrapone, 1-phenylimidazole, and piperonyl butoxide (Table III). All of these compounds (except for piperonyl butoxide up to a concentration of 1 mM) had the effect of reducing enzyme activity.

Table II. *Inhibition of kaurenoic acid hydroxylase activity by CO*

Atmosphere	Products (±SE)	Relative Oxidation
	pmol	
90% CO:10% O_2	199 ± 13	0.55
90% N_2:10% O_2	364 ± 13	1.00
80% CO:20% O_2	252 ± 29	0.69
80% N_2:20% O_2	363 ± 18	1.00

Table III. *Concentrations at which various compounds decreased kaurenoic acid hydroxylase activity by 50% (I_{50})*

Compound	I_{50}
	μM
Paclobutrazol	6.1
Tetcyclacis	9.6
BAS 111..W	19
Flurprimidol	44
Triarimol	99
Metyrapone	110
1-Phenylimidazole	430
Piperonyl Butoxide	NE[a]
Cyt *c*	5.6

[a] NE, No effect was observed for piperonyl butoxide at concentrations ranging from 1 to 1000 μM.

Solubilization of Enzyme Activity

Kaurenoic acid hydroxylase activity was readily solubilized by KCl and rapid mixing (Fig. 3). Approximately half of the total starting protein was released into the soluble fraction by 1 M KCl, and almost all of the hydroxylase activity was solubilized at this salt concentration. The specific activity of the membrane-bound hydroxylase used to produce the data shown in Figure 3 was 84 μIU mg^{-1} of protein, whereas the specific activity of the hydroxylase in the soluble fraction resulting from 1 M KCl solubilization was 196 μIU mg^{-1} of protein. Therefore, salt solubilization resulted in a 2-fold enzyme purification in this and other experiments.

DISCUSSION

The enzymes catalyzing the oxidations of kaurene to kaurenoic acid are Cyt P-450-dependent proteins (West, 1980). Cyts P-450 are a class of monooxygenases that catalyze oxidative reactions but require reducing power for activity. Associated with a Cyt P-450 is at least one additional enzyme, NADPH:Cyt P-450 reductase, an intermediate in the transfer

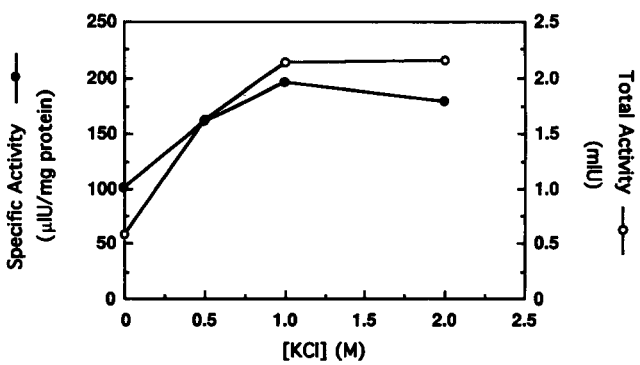


Figure 3. The specific and total activities of soluble kaurenoic acid hydroxylase as a function of the KCl concentration used for solubilization ± SE. Each enzyme assay contained 100 μL of PS_{150}, was brought to 200 mM KCl, and then was incubated for 15 min. One IU is defined as the amount of enzyme that catalyzes the formation of 1 μmol of product in 1 min at 30°C.

of electrons from NADPH to the Cyt P-450. Cyts P-450 are also characterized by their ability to bind CO in competition with O_2, which results in a loss of enzyme function. This binding can be partially reversed by 450-nm light. Earlier work demonstrated that the conversions of kaurene to kaurenol, kaurenol to kaurenal, and kaurenal to kaurenoic acid require molecular O_2 and NADPH (Dennis and West, 1967; Hasson and West, 1976). These oxidations can be inhibited by CO, and maximum reversal of this inhibition is achieved by 450 nm of illumination (Murphy and West, 1969).

The oxidation of kaurenoic acid to 7α-hydroxykaurenoic acid requires reducing potential, preferably from NADPH, and it appears to require molecular O_2 (Nakata, 1972). In the work reported here this reaction was shown to be inhibited by CO (Table II) and other Cyt P-450 inhibitors (Table III). CO binds exclusively to the ferrous heme group of a reduced Cyt P-450 (Ortiz de Montellano and Reich, 1986). Paclobutrazol, tetcyclacis, BAS 111..W, and flurprimidol are all inhibitors of GA biosynthesis that block the Cyt P-450-mediated oxidations of kaurene to kaurenoic acid (Rademacher, 1991). It is thought that these N_2-containing heterocyclic compounds have a lone pair of electrons that displace O_2 from its binding site at the protoheme iron of Cyt P-450. Triarimol, another N_2-containing heterocyclic compound, also blocks the enzymic oxidation of kaurene (Coolbaugh et al., 1982). Metyrapone and 1-phenylimidazole (common inhibitors of mammalian Cyt P-450) bind simultaneously to both the lipophilic domains and the prosthetic heme iron of Cyt P-450 (Ortiz de Montellano and Reich, 1986). All of these compounds, at micromolar concentrations, inhibited kaurenoic acid hydroxylation, further indicating that this reaction is catalyzed by a Cyt P-450.

If kaurenoic acid hydroxylase is indeed a Cyt P-450, then the stimulation of its activity by the addition of FAD (Table I) might be explained by the replacement of FAD that has dissociated from NADPH:Cyt *P*-450 reductase (Haniu et al., 1984). Furthermore, the competitive inhibition of hydroxylase activity by exogenously added Cyt *c* (Table III) is consistent with the known role of Cyt *c* as an artificial electron acceptor for the reductase. These factors, and those described above, suggest that kaurenoic acid hydroxylase is a Cyt P-450-dependent monooxygenase, similar to the enzymes immediately preceding it in the GA biosynthetic pathway.

The ability of KCl to free kaurenoic acid hydroxylase from the fungal microsome (Fig. 3) suggests that this enzyme is loosely bound to the membrane. High salt concentration was recently used to solubilize kaurene oxidase from microsomal preparations of *G. fujikuroi* (Ashman et al., 1990). Tris, phosphate, or NaCl at 400 mM resulted in the release of about 80% of the recovered activity into the soluble fraction. The necessity for detergent removal is avoided by using salts for the solubilization of these enzymes. Detergents such as sodium cholate, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, and Zwittergent 3–14 have a dramatic inhibitory effect on kaurenoic acid hydroxylase activity (our unpublished data). In addition, if the detergents are removed from the soluble fraction, then the hydroxylase and other proteins tend to aggregate, causing difficulties in enzyme purification. Whereas detergents inhibit hydroxylase activity, KCl has been shown to have a stimulatory effect on activity (Fig. 2).

Monovalent chloride salts were found to enhance the activity of the hydroxylase up to concentrations of 350 mM. It was also shown that the enhancement is an ionic effect, rather than osmotic, through assays that substituted Suc for salt at the same calculated osmotic potentials. Induction of enzyme activity by salts was previously noted by Lambeth et al. (1979) for mitochondrial Cyt P-450 involved in steroid hormone biosynthesis. Various salts, including NaCl, altered the association of two intermediate proteins required for electron transfer from NADPH to Cyt P-450: the FAD-containing reductase and an iron-sulfur protein. More recently it was shown in a filamentous fungus, *Rhizopus nigricans*, that there exists a similar Cyt P-450 system involved in steroid hydroxylation (Breskvar et al., 1987). Although this system was located in the postmitochondrial fraction, it consisted of membrane-bound Cyt P-450, reductase, and an iron-sulfur protein. The activity of another Cyt P-450 hydroxylase, purified from mammalian microsomes, was stimulated by monovalent chloride salts in the presence of Cyt b_5 (Kikuta et al., 1989). It is possible that in the kaurenoic acid hydroxylase system that the flow of electrons from NADPH to the hydroxylase is also altered by the added ions.

Now that we have partially characterized and solubilized kaurenoic acid hydroxylase from *G. fujikuroi*, we will attempt to purify this enzyme. We have had some success with ammonium sulfate fractionation, as well as gel filtration and ion exchange chromatography. There are few published reports describing the complete purification of Cyt P-450 from filamentous fungi or plants. Such purifications from plants include an unidentified Cyt P-450 from tulip bulbs (Higashi et al., 1985), *p*-chloro-*N*-methylaniline demethylase from avocado mesocarp (O'Keefe and Leto, 1989), 3,9-dihydroxypterocarpan 6α-hydroxylase from soybean (Kochs and Grisebach, 1989), and an allene oxide synthase from flaxseed (Song and Brash, 1991). Gabriac et al. (1991) recently reported using substrate-binding spectra in the presence of detergents to monitor the purification of cinnamic acid 4-hydroxylase from the tubers of manganese-induced Jerusalem artichoke. In this case, enzyme activity was not recovered after the protein had been purified to homogeneity.

Our goal in purifying kaurenoic acid hydroxylase from *G. fujikuroi* is to produce antibodies against this protein. Antibodies will be used to determine the specific intercellular location of this enzyme, and we will search for cross-reactivity between these antibodies and kaurenoic acid hydroxylase from plants. Hopefully, it will be possible to isolate the hydroxylase from a plant system by using antibodies prepared against the fungal protein. In future work we hope to purify kaurenoic acid hydroxylase from both *G. fujikuroi* and a plant system to gain a better understanding of the regulation of this important GA biosynthetic enzyme.

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