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CYTOCHROME P450-DEPENDENT HYDROXYLATION IN THE BIOSYNTHESIS OF ROSMARINIC ACID IN *COLEUS*

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Key Word Index—*Coleus blumei*; Lamiaceae; biosynthesis; rosmarinic acid; hydroxylation; cytochrome P450; cinnamic acid 4-hydroxylase; hydroxycinnamoyl-hydroxyphenyllactate 3- and 3′-hydroxylases.

Abstract—Three membrane-bound, cytochrome P450-dependent hydroxylases which are involved in the biosynthesis of rosmarinic acid have been characterized in microsomal preparations from cell cultures of *Coleus blumei*. Cinnamic acid 4-hydroxylase introduces the 4-hydroxyl group into cinnamic acid and forms 4-coumaric acid. This enzyme from *Coleus blumei* displayed saturation concentrations of 0.5 mM for both cinnamic acid and NADPH. The apparent K_m-values were determined to be at 35 and 40 μM, respectively. Hydroxycinnamoyl-hydroxyphenyllactate 3- and 3′-hydroxylases introduce the 3- and 3′-hydroxyl groups into the aromatic rings of rosmarinic acid-like esters like 4-coumaroyl-4′-hydroxyphenyllactate, 4-coumaroyl-3′,4′-dihydroxy-phenyllactate and caffeoyl-4′-hydroxyphenyllactate. 4-Coumaric acid and its CoA-ester as well as 4-hydroxyphenylpyruvate and 4-hydroxyphenyllactate were not accepted as substrates. 3-Hydroxylase was saturated with 250 μM 4-coumaroyl-3′,4′-dihydroxyphenyllactate and had an apparent K_m-value of 12.5 μM for this substrate. The respective values for 3′-hydroxylase and the substrate caffeoyl-4′-hydroxyphenyllactate were 100 and 7 μM. The order of introduction of the 3- and 3′-hydroxyl groups could not be determined. The 3- and 3′-hydroxylations are dependent on O_2 and NADPH; the saturation concentration for both enzymes for NADPH was at 0.5 mM and the apparent K_m values at 30 μM. All three hydroxylases were determined to be dependent on cytochrome P450 by inhibition experiments with cytochrome c, ancymidol, metyrapone, miconazole and tetcyclacis as well as by inhibition of the reactions in a gas phase containing CO besides O_2 and the partial reversion of this inhibition after illumination with light at 450 nm wavelength.

INTRODUCTION

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, which has two aromatic rings with two aromatic hydroxyl groups each. Since the precursors for the biosynthesis of RA are the amino acids phenylalanine and tyrosine [1], only one of the four hydroxyl groups is brought into the molecule by the precursors themselves. The other three hydroxyl groups are introduced during the biosynthesis of RA, which was completely elucidated on enzyme level in cell suspension cultures of *Coleus blumei* [2]. Phenylalanine is transformed to 4-coumaroyl-CoA by the enzymes of the general phenylpropanoid pathway, phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase (CAH) and 4-coumaroyl:CoA ligase. Cinnamic acid 4-hydroxylase, a cytochrome P450-dependent enzyme, is the first hydroxylase of the RA biosynthetic pathway introducing the 4-hydroxyl group into cinnamic acid. Tyrosine yields *p*-hydroxyphenylpyruvate by tyrosine aminotransferase [3–5], and the keto acid is reduced to 4-hydroxyphenyllactic acid by hydroxyphenylpyruvate reductase [6, 7]. RA synthase (4-coumaroyl-CoA:4-hydroxyphenyllactate 4-coumaroyl transferase), catalyses the transfer of the 4-coumaroyl-moiety of 4-coumaroyl-CoA to the aliphatic hydroxyl group of 4-hydroxyphenyllactate with the release of coenzyme A, to form 4-coumaroyl-4′-hydroxyphenyllactate [6, 8, 9]. With caffeoyl-CoA and 3,4-dihydroxyphenyllactic acid this enzyme can directly form RA. The hydroxyl groups in positions 3 and 3′ of the aromatic rings of 4-coumaroyl-4′-hydroxyphenyllactate are successively introduced by two membrane-bound hydroxylases, thus giving rise to RA. In this paper the three hydroxylases involved in the biosynthetic pathway of RA are characterized as cytochrome P450-dependent hydroxylases.

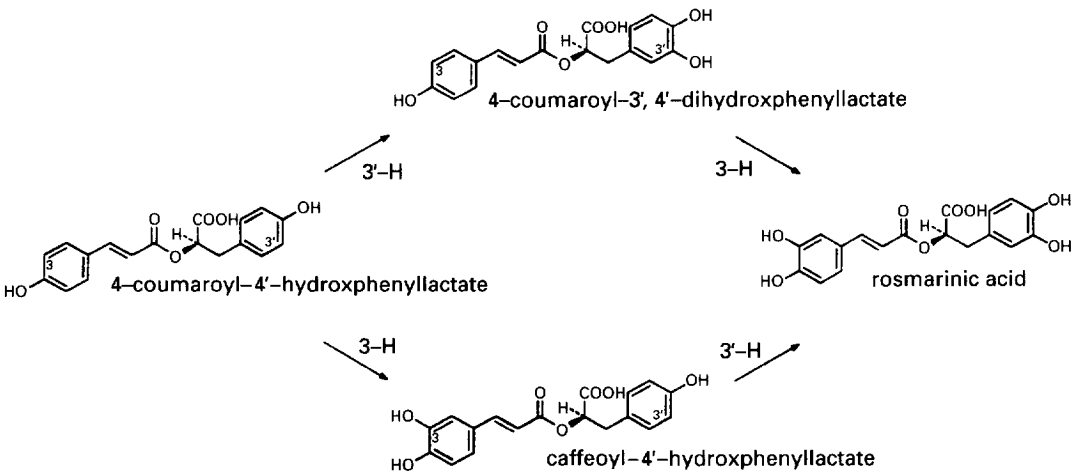

Fig. 1. 3- and 3′-hydroxylation reactions involved in rosmarinic acid biosynthesis. The sequence of hydroxylations in the biosynthetic pathway of rosmarinic acid could not yet be determined.

RESULTS

Identification of CAH and hydroxycinnamoyl-hydroxyphenyllactate 3- and 3′-hydroxylases

The general phenylpropanoid metabolism forming 4-coumaroyl-CoA from phenylalanine is part of the biosynthetic pathway of RA [2]. One of the involved enzymes is cinnamic acid 4-hydroxylase (CAH), a cytochrome P450-dependent monooxygenase. CAH activity can be detected in microsomal preparations from cell cultures of *Coleus blumei*, introducing the hydroxyl-group into cinnamic acid in a *para*-position to the side chain.

In the same microsomal preparations, hydroxylating activities were detected which introduce hydroxyl groups in positions 3 and 3′ of the aromatic rings of RA-like esters (4-coumaroyl-3′,4′-dihydroxyphenyllactate, 4-coumaroyl-4′-hydroxyphenyllactate, caffeoyl-4′-hydroxyphenyllactate). These RA-like esters are formed *in vitro* from differently substituted precursors (4-coumaroyl-CoA, caffeoyl-CoA, 4-hydroxyphenyllactate, 3,4-dihydroxyphenyllactate) by rosmarinic acid synthase (RAS), the ester-forming enzyme in the biosynthetic pathway of RA. The reaction product formed in the cells of *Coleus blumei* is supposed to be 4-coumaroyl-4′-hydroxyphenyllactate [2]. The consecutive action of the 3- and 3′-hydroxylases results in the formation of RA (Fig. 1), which was identified by HPLC with help of authentic RA. These hydroxylases showed specificity for RA-like esters, since neither 4-coumaric acid, 4-coumaroyl-CoA, 4-hydroxyphenylpyruvate nor 4-hydroxyphenyllactate were hydroxylated in position 3 of their aromatic rings. These hydroxylases were identified as hydroxycinnamoyl-4′-hydroxyphenyllactate 3′-hydroxylases (3′-hydroxylase, 3′-H) and 4-coumaroyl-hydroxyphenyllactate 3-hydroxylase (3-hydroxylase, 3-H).

Inhibitor studies

Several inhibitors reported to be specific for phenolases or for cytochrome P450-dependent monooxygenases were tested for their effects on all three hydroxylase activities. In enzyme preparations from *Coleus blumei* cells phenolase activities can be detected with a number of different substrates, e.g. 4-coumaric acid or 4-hydroxyphenyllactate [10]. These phenolases can introduce the 3- and 3′-hydroxyl groups into RA-like esters as well, but their activities are completely inhibited after addition of 1 mM of the phenolase inhibitors salicylhydroxamic acid or diethyldithiocarbaminate (M. Petersen, unpublished results). After addition of 1 or 10 mM diethyldithiocarbaminate or 1 mM salicylhydroxamic acid to microsomal preparations from *Coleus blumei* cells, in order to inhibit residual contaminating phenolase activities, the activities of 3-H and 3′-H were found to be unaffected or even slightly enhanced in comparison with the assays without phenolase inhibitors. The activity of CAH was similarly influenced by these inhibitors. This indicates that the 3- and 3′-hydroxylations of RA-like esters in microsomal preparations from cells of *Coleus blumei* are not due to phenolase activities.

Potassium cyanide, an inhibitor of peroxidases [11] had no relevant inhibitory effect on the hydroxylation reactions of CAH, 3-hydroxylase and 3′-hydroxylase up to a concentration of 1 mM. Cytochrome c, an electron acceptor competing for electrons transferred from NADPH:cytochrome P450 reductase to cytochrome P450, strongly inhibited CAH, 3-H and 3′-H at a concentration of only 10 μM. Ancymidol, metyrapone, miconazole, juglone and tetcyclacis are reported to be inhibitors for cytochrome P450-dependent enzymes [12–15]. At concentrations ranging from 0.1 to 2 mM all inhibitors influenced the three hydroxylase activities negatively (Fig. 2). In all cases 3-H and 3′-H were inhibited more severely than CAH.

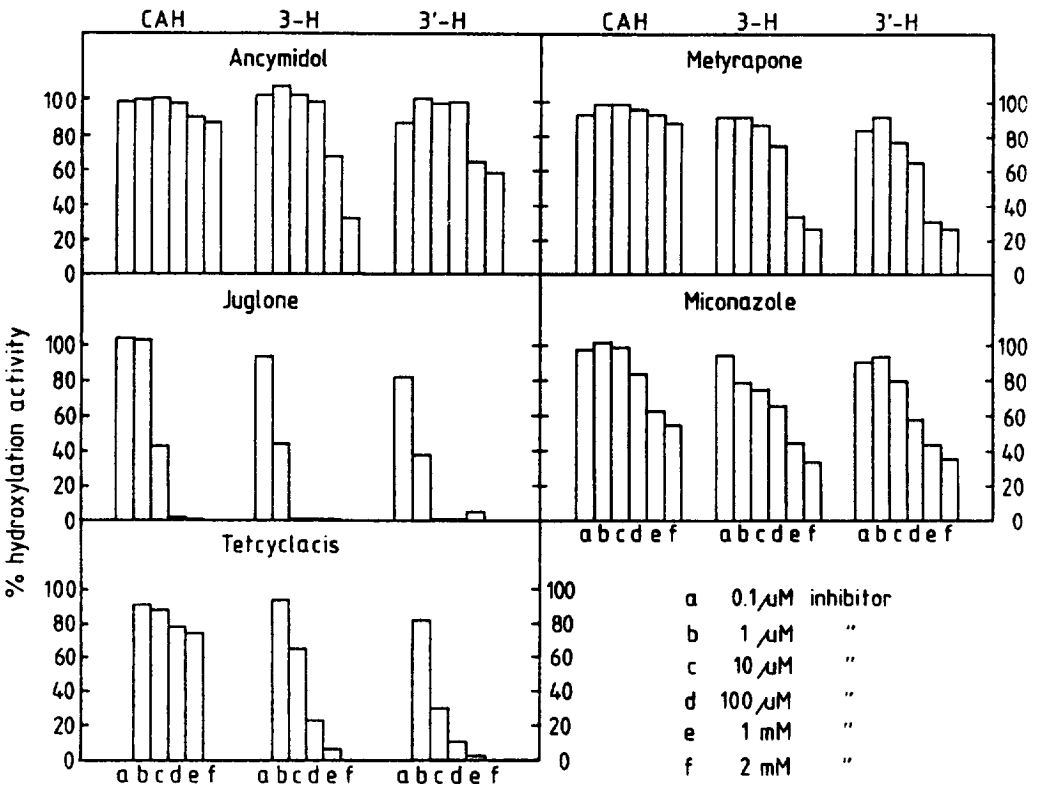

Fig. 2. Influence of different cytochrome P450 inhibitors on the activities of CAH, 3'-hydroxylase (3-H) and 3-hydroxylase (3'-H). A specific activity of 100% is equivalent to 64 µkat kg^{-1} (CAH), 13 µkat kg^{-1} (3'-H), 20 µkat kg^{-1} (3-H) for the experiments with ancymidol, metyrapone, juglone and miconazole, and 24 µkat kg^{-1} (CAH), 20 µkat kg^{-1} (3'-H), 22 µkat kg^{-1} (3-H) for experiments with tetcyclacis.

The strongest inhibitor seemed to be juglone; in our case, however, juglone was found to bind strongly to phenolic compounds and to precipitate them. Reaction substrates and products were therefore eliminated from the assays, and the observed inhibition was not due to a real enzyme inhibition. The effect of the other four chemicals, which are used as antifungal substances or plant growth retardants, strongly suggests the participation of cytochrome P450 in all three hydroxylation reactions.

Properties of CAH, 3-hydroxylase and 3'-hydroxylase

All three hydroxylase activities involved in the biosynthetic pathway of RA were determined in the microsomal fraction after $MgCl_2$-precipitation and centrifugation at 48 000*g*. Except phenolase activities the desalted supernatant of this centrifugation step contained no hydroxylating activities. CAH, 3-hydroxylase and 3'-hydroxylase therefore are membrane-bound enzymes.

In the standard assay the reaction yields of CAH and 3-H were linear with the concentration of microsomal protein up to 400 µg ml^{-1}, and the reaction yield of 3'-H up to 300 µg ml^{-1}. Time linearity was achieved in standard assays up to 50 min for 3-H and 20 min for 3'-H and CAH.

All three hydroxylases showed a pH-optimum of pH 7.5 in Tris–HCl-buffer. The curves of the pH-dependence of 3-H was a little steeper with half-maximal activities at pH 6.25 and 8.2 than the curves for of CAH and 3'-H with half-maximal activities at pH 6.6 and 8.6, and pH 6.1 and 8.1, respectively.

The temperature optima of CAH, 3-H and 3'-H were around 25°. Whereas 3'-H quickly lost activity at higher temperature, 3-H retained high activities up to 35° before its activity declined at higher temperatures.

All three hydroxylases showed higher activities when dithiothreitol was added to the reaction assay. The optimal concentrations were 10 mM for CAH, 1 mM for 3'-H and 0.5 mM for 3-H. However, routinely 3-H and 3'-H were assayed without dithiothreitol.

Michaelis–Menten-kinetics without any substrate inhibition were observed for all three hydroxylases. CAH was saturated at about 0.5 mM cinnamic acid; the apparent K_m-value for this substrate was at 35 µM. The activity of 3-H was routinely measured with 4C-DHPL (4-coumaroyl-3',4'-dihydroxyphenyllactate = *R*(+)-2-(4-hydroxycinnamoyloxy)-3-(3',4'-dihydroxyphenyl)propionic acid) as substrate. This leads to the formation of RA. The enzyme was saturated at 250 µM of this substrate; the apparent K_m-value was at 12.5 µM. 3-H most probably also accepts 4C-*p*HPL [4-coumaroyl-4'-hydroxyphenyllactate = *R*(+)-2-(4-hydroxycinnamoyloxy)-3-(4'-hydroxy-

phenyl)propionic acid] as substrate, but since this is also a substrate for 3′-H, the two hydroxylases compete for this substrate and the K_m-values for both enzymes for this substrate cannot be determined at this stage of enzyme purification. After application of 4C-*p*HPL and NADPH to microsomal preparations of *Coleus blumei* cells, Caf-*p*HPL (caffeoyl-4′-hydroxyphenyllactate = *R*(+)-2-(3,4-dihydroxycinnamoyloxy)-3-(4′-hydroxypenyl)propionic acid) was formed with much higher activity than 4C-DHPL. The activity of 3′-H was measured with Caf-*p*HPL as substrate; this again leads to the formation of RA. The enzyme was saturated at 100 μM Caf-*p*HPL; the observed apparent K_m-value was at 7 μM. The reported saturation concentrations and K_m-values for 3-H and 3′-H were calculated with molar extinction coefficients for 4C-*p*HPL, 4C-DHPL and Caf-*p*HPL enzymatically prepared with help of RA synthase in very low amounts [9]. For these substrates preliminary extinction coefficients were determined spectrophotometrically (M. Petersen, unpublished results) and used for the calculation of substrate concentrations. The K_m-values therefore should be regarded as preliminary values.

The very low K_m-values of 3-H and 3′-H for 4C-*p*HPL and Caf-DHPL, respectively, show the high affinity of these hydroxylases for RA-like esters, even when the natural substrate for one of the hydroxylases should be 4C-*p*HPL. The order of the introduction of the 3- and 3′-hydroxyl groups could not be determined at this stage of purification, since 3-H and 3′-H occur in the same microsomal preparation and up to now no specific inhibitor for one of the hydroxylases was found.

Dependence of hydroxylases on cofactors

The reactions of CAH, 3-H and 3′-H are dependent on NADPH as cosubstrate (Table 1). With NADH as electron donor CAH showed no activity at all, whereas 3-hydroxylase and 3′-hydroxylase had only 10% or less of the hydroxylation activities measured with NADPH. Simultaneous addition of 0.2 mM NADPH and 0.2 mM NADH did not lead to a prominent synergistic effect of these two reducing equivalents, as observed for some cytochrome P450-dependent reactions [13, 16–19]. This might indicate that cytochrome b_5 is not involved in the electron transfer to the different cytochrome P450s of the CAH, 3-H and 3′-H reactions as postulated for animal cytochrome P450s by Estabrook *et al.* [20]. Addition of NADP together with NADH in presence or absence of ATP did not lead to the hydroxylation of cinnamic acid or RA-like esters showing that transhydrogenases were not active in microsomal preparations of *Coleus blumei* cells. NADPH:cytochrome P450 reductase is known to contain FAD and FMN as prosthetic groups. These flavine nucleotides might be partially lost during enzyme preparation and purification. Addition of FAD to the hydroxylase assay enhanced the activities of CAH, 3′-H and 3-H by 13, 29 and 16%, respectively.

Table 1. Influence of NAD(H), NADP(H) and FAD on the activities of CAH, 3-hydroxylase (3-H) and 3′-hydroxylase (3′-H)

Cosubstrate	% Activity CAH	3′-H	3-H
0.5 mM NADPH	100	100	100*
0.2 mM NADPH	97	94	85
0.5 mM NADH	0	9	10
0.2 mM NADH	0	8	6
0.5 mM NADP	0	1	7
0.2 mM NADP	0	0	1
0.2 mM NADPH + 0.2 mM NADH	93	104	93
0.2 mM NADP + 0.2 mM NADH	0	0	4
0.2 mM NADP + 0.2 mM NADH + 0.1 mM ATP	0	6	4
1 mM NADPH	100	100	100†
1 mM NADPH + 5 μM FAD	113	129	116

* Specific activity of 100% = 29 μkat kg^{-1} (CAH), 24 μkat kg^{-1} (3′-H), 39 μkat kg^{-1} (3-H).

† Specific activity of 100% = 4 μkat kg^{-1} (CAH), 9 μkat kg^{-1} (3′-H), 15 μkat kg^{-1} (3-H).

The determination of the dependence of CAH, 3-H and 3′-H upon NADPH revealed normal Michaelis–Menten-kinetics for all three enzymes. They were saturated at an NADPH concentration of 0.5 mM. The apparent K_m-value for CAH was at 40 μM, the values for 3-H and 3′-H were at 30 μM.

CO inhibition and reversion by blue light

A typical feature of cytochrome P450-dependent enzymes is the inhibition of their reaction in an atmosphere containing CO besides O_2 and the at least partial reversion of this inhibition after illumination with light of the wavelength 450 nm [21]. After treating hydroxylase assays with N_2 for 20 min only residual activities of 9% (CAH), 33% (3′-hydroxylase) and 27% (3-hydroxylase) could be detected. Assays containing an O_2-consuming system (50 mM glucose, 167 nkat glucose oxidase, 334 nkat catalase) showed even lower hydroxylase activities (Table 2). As typical for cytochrome P450-dependent enzymes, the reactions of CAH, 3-H and 3′-H were inhibited in an atmosphere consisting of 20% CO, 17% O_2 and 63% N_2 by 96, 52 and 68 %, respectively. All thee activities were considerably higher when the reaction assays were illuminated with 450 nm-light (Table 2). This CO-inhibition and its partial reversion by blue light suggests the participation of cytochrome P450 in all three hydroxylation reactions involved in the biosynthetic pathway of RA.

Table 2. Influence of different gas phases and illumination with 450 nm-light on the activities of CAH, 3-hydroxylase (3-H) and 3′-hydroxylase (3′-H)

Treatment	Activity [%] CAH	3′-H	3-H
Control (air)	100	100	100*
N_2	9	33	27*
20% CO + 17%O_2 + 63%N_2, dark	4	32	48*
20% CO + 17%O_2 + 63%N_2, 450 nm	42	51	70*
Control + O_2-consuming system†	8	9	19‡

*mean of six independent experiments: 100% = 37 μkat kg^{-1} (CAH), 9 μkat kg^{-1} (3′-H), 12 μkat kg^{-1} (3-H).

† O_2-consuming system: 50 mM glucose, 167 nkat glucose oxidase, 334 nkat catalase.

‡ mean of five independent experiments: 100% = 23 μkat kg^{-1} (CAH), 6 μkat kg^{-1} (3′-H), 13 μkat kg^{-1} (3-H).

DISCUSSION

Many secondary compounds derived from the general phenylpropanoid pathways show typical substitution patterns at their aromatic rings. Usually, besides the 4-OH group one or two hydroxyl or methoxy groups in positions 3 and 5 are found at the phenylalanine-derived aromatic ring(s). This is the case for chlorogenic acid, caffeoyl-shikimate, flavonoids and isoflavonoids. Other aromatic rings in more complex compounds stemming from other precursors show characteristic hydroxylation or methoxylation patterns as well. The 4-hydroxyl group of phenylalanine-derived compounds is introduced by cinnamic acid 4-hydroxylase, a cytochrome P450-dependent monooxygenase which is a specific enzyme of the general phenylpropanoid metabolism. This enzyme was first detected and described from pea seedlings [22, 23]. The introduction of the 3-hydroxyl group is an unclarified question for many secondary compounds having a caffeoyl-moiety, since the first description of the general phenylpropanoid pathway. The participation of phenolases which are capable hydroxylating aromatic compounds in the *ortho*-position to an existing OH-group has been discussed for a long time [24–27]. Phenolases are localized in plastids [28] and are considered to be rather unspecific, accepting a great variety of different phenolic substrates. Phenolases hydroxylating 4-coumaric acid derivatives were described, e.g from potato [26] and sweet potato [29]. The complete inhibition of phenolase activities from *Vigna mungo* by tentoxin, however, did not alter the content of caffeic acid derivatives [30]. This was taken as a proof that a 4-coumaric acid hydroxylase had to be present, which is not a phenolase. A specific hydroxylation of 4-coumaric acid or 4-coumaroyl-CoA by enzymes other than phenolases has not been described frequently. Kamsteeg *et al.* [31] described a soluble 4-coumaroyl-CoA hydroxylase needing NADPH, FAD and O_2 from petals of *Silene dioica*; 4-coumaric acid was not accepted as substrate. Particulate preparations from potato tubers introduced the 3-hydroxyl group into 4-coumaric acid or tyrosine [32]; this enzyme was dependent on NAD(P)H and FAD/FMN. It showed some properties of phenolases, but exhibited a quite strong substrate specificity and could not use ascorbate as reductant. A Zn^{2+}-dependent 4-coumaroyl-CoA hydroxylase from parsley was activated by pH-shift and used ascorbate as reductant [33]. Kojima and Takeuchi [34] described a 4-coumaric acid hydroxylase in particulate preparations from *Vigna mungo* seedlings which used a wide variety of reducing compounds, but preferred ascorbate. The enzyme was considered not to be a typical phenolase, since it was active in preparations in which typical phenolase activities had been eliminated by the fungal toxin tentoxin. A cytochrome P450-dependent monooxygenase was found to catalyse the 5-hydroxylation of ferulic acid to 5-hydroxyferulic acid during lignin biosynthesis in *Populus Xeuamericana* [17].

The preceding paragraph shows that a general mechanism for the biosynthesis of caffeic acid and its derivatives as well as further hydroxylated or methoxylated compounds in higher plants cannot be found. Since simple cinnamic acid-derived compounds and lignins occur in every higher plant, the occurrence of a general hydroxylation mechanism acting on the level of C_6–C_3-compounds could be anticipated. The substitution pattern of the aromatic rings of phenylpropanoid derivatives can be introduced rather late during the biosynthetic pathway, namely after the completion of the carbon skeleton of the respective compounds. This is the case for, e.g. chlorogenic acid or caffeoyl-shikimic acid [18, 35], flavonoids [16, 36–40] and isoflavonoids [13]. For all these compounds the biosynthetic scheme followed is the use of activated *p*-coumaric acid derivatives as precursors for the formation of the more complex carbon skeleton and the completion of the substitution pattern of the aromatic ring(s) by introduction of the 3-hydroxyl groups at late biosynthetic steps. The respective enzymes involved in flavonoid and isoflavonoid biosynthesis all are cytochrome P450-dependent enzymes. For the biosynthesis of chlorogenic acid it has not yet been clarified whether the second hydroxyl group at the aromatic ring is introduced before or after esterification of the hydroxycinnamic acid with quinic acid. However, Kühnl *et al.* [35] described a cytochrome P450-dependent monooxygenase from carrot cell suspensions hydroxylating *trans*-5-*O*-(4-coumaroyl)-D-quinate to chlorogenic acid; this enzyme also accepted *trans*-5-*O*-(4-coumaroyl)-D-shikimate, but not 4-coumaric acid as substrates. A similar enzyme was described from parsley cell cultures by Heller and Kühnl [18].

Up to now, most enzymes introducing the substituting OH-groups into the phenylalanine-derived parts of more complex natural compounds have been found to be dependent on cytochrome P450. The same is true for the biosynthesis of RA, where also the substitution patterns of the aromatic rings, in this case derived from phenylalanine and tyrosine, are com-

pleted by cytochrome P450-dependent 3- and 3′-hydroxylations. These two enzyme activities therefore add to the list of cytochrome P450-dependent hydroxylations involved in plant secondary metabolism and especially in the introduction of substituting OH-groups. The 3- and 3′-hydroxylase activities were isolated and characterized together with the well-known cytochrome P450-monooxygenase cinnamic acid 4-hydroxylase from cell-suspension cultures of *Coleus blumei* accumulating RA. Inhibitor studies showed that all three hydroxylase activities are not influenced by phenolase and peroxidase inhibitors, whereas typical cytochrome P450 inhibitors like ancymidol, miconazole, metyrapone and tetcyclacis and especially cytochrome c strongly inhibit all three reactions. This behaviour, together with the inhibition of all three reactions in a gas phase containing CO besides O_2 and the partial reversion of this inhibition by 450 nm light prove that CAH, 3-H and 3′-H are cytochrome P450-dependent enzymes. The other enzyme characteristics are also typical for this type of hydroxylases. The reactions are dependent on molecular oxygen and NADPH. NADH cannot replace NADPH as electron donor. The hydroxylation reactions can be stimulated by addition of FAD which is an essential component of NADPH:cytochrome P450 reductase. All three hydroxylases show very similar behaviour towards their substrate NADPH with respect to substrate saturation concentration and apparent K_m-values. The different cytochrome P450s might be served with electrons by the same population of NADPH:cytochrome P450 reductase. Nowadays it is believed that not every cytochrome P450 has its own type of reductase. From Jerusalem artichoke only three closely related forms of NADPH:cytochrome P450 reductase could be resolved [41], although a higher number of cytochrome-P450 hydroxylases must be active in this plant.

Several data on the 3- and 3′-hydroxylations in the biosynthetic pathway of RA indicate that the two reactions are catalysed by separate cytochrome P450-monooxygenases. The influence of the temperature on the 3- and 3′-hydroxylases was different, 3′-H being more sensitive against higher temperatures. The enzymes involved in 3- and 3′-hydroxylations of RA-like esters are differently sensitive towards the cytochrome P450 inhibitor tetcyclacis applied *in vivo* to the suspension cultures. Under these conditions, caffeoyl-4′-hydroxyphenyllactate accumulates in the cells besides RA, showing a higher sensitivity of 3′-H (data not shown). Interestingly, a selected cell line of *Coleus blumei* also accumulates high amounts of caffeoyl-4′-hydroxyphenyllactate besides RA (M. Petersen, unpublished results).

3-H and 3′-H are very specific towards their aromatic substrates, accepting only RA-like esters as substrates. Precursors of the RA biosynthetic pathway like 4-coumaric acid, 4-coumaroyl-CoA, 4-hydroxyphenylpyruvate and 4-hydroxyphenyllactate are not hydroxylated by these enzymes. Although three enzymes of the earlier part of RA biosynthesis accept monohydroxylated as well as dihydroxylated substrates, namely hydroxycinnamic acid:CoA ligase, hydroxyphenylpyruvate reductase and RA synthase, the existence of specific enzymes introducing the 3- and 3′-hydroxyl groups of the two aromatic rings only at the ester stage makes it improbable that in the cell the introduction of these 3-hydroxyl groups would normally occur at the 'monomer' stage, i.e. the stage of the phenylalanine- and tyrosine-derived ester precursors. Therefore, RA biosynthesis is another example, where the final substitution pattern of the aromatic rings of the natural product is established rather late in the biosynthetic pathway, after the completion of the carbon skeleton.

EXPERIMENTAL

Cell cultures. Cell suspension cultures of *Coleus blumei* were initiated from a callus culture, which was kindly provided by Dr B. Ulbrich (Rhône–Poulenc–Rorer, formerly Nattermann, Cologne), in CB2-medium and subcultured as described previously [6]. For isolation of enzymes the cells were cultivated in CB4-medium (4% sucrose).

Preparation of microsomal fraction. Cells were harvested after 8 days of cultivation by suction filtration. All further steps were performed at 0–4°. Cells were ground in a mortar with 0.2 g Polyclar 10 and 1 ml buffer (0.1 M Tris–HCl, 1 mM dithiothreitol (DTT), 1 mM diethyldithiocarbaminate (DIECA), pH 7.5) per g fresh mass. The homogenate was filtered through nylon mesh and centrifuged at 8000*g* for 20 min. The supernatant was used for $MgCl_2$-pptn of microsomal membranes. It was adjusted to 50 mM $MgCl_2$ and stirred on ice for 20 min. After centrifugation at 48 000*g* for 20 min the sediment was resuspended in buffer (as above, but without DTT) with help of a glass homogenizer (Potter and Elvehjem) and used for enzyme assays.

Determination of protein concentrations. Protein concs were determined according to ref. [42] with bovine serum albumin as standard.

Enzyme assays. Hydroxylase activities were routinely determined in Eppendorf vials in a total vol of 500 μl 0.1 M Tris–HCl buffer pH 7.5. For some purposes all given vols were halved. Assays for CAH activity contained 1.3 mM cinnamic acid (stock soln 50 mM in 50% EtOH), 10 mM DTT, 1 mM DIECA, 0.5 mM NADPH and 0.25 mg microsomal protein. Assays for 3- and 3′-hydroxylases contained 0.25 mg microsomal protein, 1 mM DIECA, 0.5 mM NADPH and 0.2 mM 4C-DHPL or 0.1 mM Caf-pHPL (prepd enzymatically according to ref. [9]), respectively. Assays were routinely incubated at 25° for 10 min, stopped by addition of 100 μl 6 M HCl and cooling on ice.

Identification and quantification of reaction products. Reaction products were extracted by mixing stopped enzyme assays ×3 with 0.5 ml EtOAc each, cen-

trifugation and collection of the EtOAc phases, which were combined and evapd to dryness under vacuum. The residues were redissolved in 75 μl MeOH–0.01% H_3PO_4 and centrifuged after addition of 75 μl 0.01% H_3PO_4 in H_2O. Analysis and quantification of the reaction products was performed by HPLC by using a Hypersil ODS column (40 × 4.6 mm + 250 × 4.6 mm, particle size 5 μm) and isocratic elution with 50% MeOH–50% H_2O acidified with 0.01% H_3PO_4 (for 3- and 3′-hydroxylase assays) or 45% MeOH–55% H_2O acidified with 0.01% H_3PO_4 (for 4-coumaric acid). Eluting substances were monitored spectrophotometrically at 333 nm (for 3- and 3′-hydroxylase assays) or 309 nm (for CAH assays). A defined concn (25 μM) of authentic 4-coumaric acid or RA was used as external standard for quantification.

CO-inhibition experiments. Gases (CO, N_2, synthetic air) were mixed in a separatory funnel (500 ml) and pumped to the reaction vessels (2 ml screw-capped glass vials with rubber–Teflon septum) containing all the reaction components except NADPH by a peristaltic pump. The vials were placed on ice and the gas mixts were bubbled through the liquid for 15 min through capillaries. After that the capillaries were sealed and the reaction vessels were warmed to 20°. After addition of NADPH by a syringe through the septum the enzyme reactions were incubated at 20° for 15 min in the dark or illuminated with 450 nm-light with help of a slide projector and a Schott colour glass combination SFK 7. The reactions were stopped, extracted and analysed as described.

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