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Formation of *trans*-Caffeoyl-CoA from *trans*-4-Coumaroyl-CoA by Zn^{2+}-Dependent Enzymes in Cultured Plant Cells and Its Activation by an Elicitor-Induced pH Shift

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A novel hydroxylase activity catalyzing the formation of *trans*-caffeoyl-CoA from *trans*-4-coumaroyl-CoA was identified in crude extracts from cultured parsley cells. The extracts were less active (V_{max}/K_m) in converting *trans*-4-coumaric to *trans*-caffeic acid. Optimal hydroxylase activity was found at pH 6.5 with a steep decline toward both pH 7.4 and pH 5.0. The enzyme activity requires ascorbate and Zn^{2+} at optimal concentrations of 50 and 0.5 mM, respectively. No other reductant could replace ascorbate, whereas high concentrations of Ca^{2+} partially substituted for Zn^{2+}. The enzyme is soluble and appears to be located in the cytoplasm. The unusual pH optimum suggests that the hydroxylase is inactive at the normal cytoplasmic pH. Upon treatment of parsley cells with an elicitor derived from *Phytophthora megasperma* f. sp. *glycinea*, the cytoplasmic pH dropped by approximately 0.25 pH unit within 55 min as determined by ^{31}P NMR spectroscopy. Our results suggest that this shift in the cytoplasmic pH is sufficient for the activation of the hydroxylase, eventually leading to the formation of caffeoyl and feruloyl esters. Such esters may be a part of a very rapid resistance response of the plant cells, which would leave no time for *de novo* enzyme synthesis.

A number of factors play an important role in a plant's defense against potential pathogens. Among these are preformed physical and chemical barriers such as cutin or phenolic substances. On the other hand, plant resistance may also be the result of induced defense mechanisms in which metabolic processes in the plant cells are drastically changed upon contact with the potential pathogen (1). Known induced defense mechanisms include cell wall modifications due to the synthesis of callose and phenolic complexes such as lignin or suberin, the accumulation of hydroxyproline-rich proteins in the cell wall, and the accumulation and excretion of phytoalexins (2-6). The activation of most disease resistance mechanisms is thought to be due primarily to the transcriptional induction of the relevant genes (7-10). However, it has been shown that the β-1,3-glucan synthase responsible for callose biosynthesis, for example, is activated by a local increase in cytoplasmic Ca^{2+} concentration (11). Other intracellular factors such as shifts in pH (12) and the distribution of inorganic phosphate (13) have also been suggested as being involved in the induction of defense responses.

The hydroxylation of 4-coumaric acid to caffeic acid is a central reaction in the synthesis of lignin and other phenolic substances. The catalysis of this reaction has often been ascribed to a phenolase enzyme which requires molecular oxygen and a re-

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ductant such as ascorbate or NAD(P)H[2] (14). More recently, the hydroxylation of 4-coumaroyl-CoA by an FAD-dependent enzyme activity from *Silene dioica* petals has been reported (15). In some systems, for example parsley cells, this activity is lacking, and it has been suggested that the cellular requirement for caffeoyl moieties is covered by the hydroxylation of *trans*-5-*O*-(4-coumaroyl)shikimate catalyzed by a cytochrome P450-dependent monooxygenase (16).

In this report we describe a novel enzyme activity from cultured parsley cells which hydroxylates 4-coumaroyl-CoA to caffeoyl-CoA. This enzyme activity was also found in various other cultured plant cells. Its widespread occurrence and its regulation by small pH changes in the physiological pH range make this enzyme a prime candidate for the initiation of the biosynthesis of polyphenolic substances in infected plant tissue.

MATERIALS AND METHODS

Materials. All chemicals were of analytical grade. [2-^{14}C]Malonic acid (603.5 GBq/mol) was purchased from Amersham-Buchler, Braunschweig, West Germany. 4-Coumaric acid, caffeic acid, chlorogenic acid, sodium ascorbate, 4-nitrophenyl phosphate, and 4-hydroxybenzaldehyde were from Sigma-Chemie, Deisenhofen, West Germany; coenzyme A, NADPH, NADH, FAD, and FMN were purchased from Boehringer, Mannheim, West Germany. *trans*-4-Coumaroyl-CoA and *trans*-caffeoyl-CoA were synthesized according to Stöckigt and Zenk (17). *trans*-4-Coumaroylshikimate, *trans*-caffeoylshikimate, and *trans*-coumaroylquinate were all kindly supplied by U. Koch, Freiburg.

Induction of cell suspension cultures. Elicitor was prepared from the cell wall of *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*) as described elsewhere (18). Parsley cell suspension cultures were grown in the dark as described previously (19), and the elicitor (4 mg) was added to these cultures (40 ml) in a sterile aqueous solution (1 ml). The cells were harvested by filtration after various periods of time (3–18 h), immediately frozen in liquid nitrogen, and stored at −70°C until required.

Chromatography. Thin-layer chromatography on cellulose plates (Merck, Darmstadt, West Germany) was carried out in the following solvents: (I) 1 M boric acid/sodium borate, pH 7; (II) 1-butanol/acetic acid/water (5/2/3, v/v/v; or (III) 2% formic acid. Chloroform/acetic acid/water (5/1/1, v/v/v, upper phase) was used as the solvent (IV) with silica gel plates (Merck, Darmstadt, West Germany). Paper chromatography was carried out using solvent (II) on Whatman 3MM paper which had been prewashed successively in 10 mM EDTA in water, water, and methanol. HPLC was performed on a Lichrosorb RP-18 (5 μm) column (Knauer, Bad Homburg, West Germany) using 50 mM sodium citrate, pH 4.6/methanol (65/35, v/v) at a flow rate of 1 ml/min.

Buffers. The following buffers, all containing 50 mM ascorbate, were used: (A) 50 mM Mes, pH 6.5; (B) 50 mM Mes/Mops, pH 6.4–7.5; (C) 100 mM ammonium acetate, pH 4.5–6.5.

Preparation of enzyme extracts. All procedures were carried out at 4°C. Frozen cells (10 g) were homogenized in a mortar with Dowex 1x2 (1 g) and buffer A (10 ml). The homogenate was filtered through a nylon screen (70 μm opening) and cleared by centrifugation (15 min; 20,000*g*), and the supernatant was desalted on a Sephadex G-25 column equilibrated in buffer A.

Enzyme assays. The standard assay for 4-coumaroyl-CoA 3-hydroxylase[3] activity contained 150 mM [β-^{14}C]4-coumaroyl-CoA (188 GBq/mol), 500 μM $ZnSO_4$, and 20 μg protein in buffer A (total volume 50 μl). The mixture was incubated for 30 min at 30°C, and the reaction was terminated by the addition of 5.5 μl 5 M NaOH. After hydrolysis (15 min, 40°C) and acidification, the hydroxycinnamic acids were extracted into ethyl acetate (100 μl) and separated by TLC. All operations were carried out in the dark to minimize *cis-trans* isomerization. Radioactivity on the plates was located and integrated with an LB 2832 TLC analyzer (Berthold, Wildbad, West Germany). For HPLC analysis, the extracted hydroxycinnamic

[2] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; PAL, phenylalanine ammonia-lyase; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); NADH, nicotinamide adenine dinucleotide (reduced form); FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Pipes, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; *Pmg*, *Phytophthora megasperma* f. sp. *glycinea*.

[3] Enzymes: monophenol monooxygenase, EC 1.14.18.1; 4-hydroxyphenylpyruvate dioxygenase, EC 1.13.11.27; 4-hydroxyphenyl-pyruvate hydroxylase; phenylalanine ammonia-lyase, EC 4.3.1.5; *trans*-4-coumaroyl-CoA 3-hydroxylase.

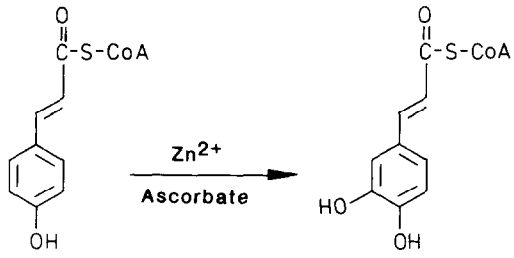

FIG. 1. Reaction catalyzed by the *trans*-4-coumaroyl-CoA 3-hydroxylase from cultured plant cells.

acid mixture was evaporated to dryness and redissolved in 20 μl for sample injection (Waters, Königstein). Phenylalanine ammonia-lyase activity was measured according to Ref. (20).

Vacuole enrichment. Vacuoles from cultured parsley cells were isolated by lysis of protoplasts as described previously (21). Acid phosphatase activity (22) served as a vacuole marker.

^{31}P NMR measurements. Parsley cells were cultivated for 2 weeks in manganese-free B5 medium (23), harvested by filtration, and washed extensively with manganese- and phosphate-free B5 medium. The cells (approx 6 ml packed volume) suspended in the latter medium (4 ml) were transferred to an NMR tube (20 mm i.d.). ^{31}P NMR spectra were recorded at 121.49 MHz on a Bruker MSL 300 spectrometer (Rijksuniversiteit te Utrecht). Radiofrequency pulses of 50 μs (60° tip angle) were used, and the recycling time was 0.5 s. Spectra were accumulated in blocks of 750 scans (approx 6 min). Methylenediphosphonic acid served as the external standard (13). Aerobic conditions during NMR spectroscopy were maintained by stirring the suspension with an air-driven mixer while applying a stream of air to the surface. Anaerobic suspensions were created by replacing the air supply with nitrogen.

Protein determination. Protein was measured according to Bradford with bovine serum albumin as the standard (25).

RESULTS

4-Coumaroyl-CoA 3-Hydroxylase Activity

When *trans*-[β-^{14}C]4-coumaroyl-CoA was incubated with a crude cell extract from parsley cells in buffer A, a radioactive product could be separated by paper chromatography or cellulose TLC in solvent (III). The product was identified as *trans*-caffeoyl-CoA (Fig. 1) by chromatographic comparison with the authentic reference compound and by its bright blue fluorescence under ultraviolet irradiation (254 nm). Furthermore, caffeic acid released from the enzymatic product upon hydrolysis was identified by TLC in various solvent systems and by reversed-phase HPLC.

The enzyme activity of crude extracts was strongly dependent on the pH as well as on the buffer composition. Almost no activity could be detected in phosphate buffers. Furthermore, the activity was lost completely on Sephadex G-25 gel filtration prior to the incubation, but could be partially restored by recombining the eluates. The low-molecular-weight components necessary for restoration of the enzyme activity were identified as Zn^{2+} and ascorbate. NADPH, NADH, reduced glutathione, and dimethyltetrahydropterine could not substitute for ascorbate, whereas Zn^{2+} could be partially replaced by high concentrations of Ca^{2+} but not by BO_3^{3-}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Fe^{2+} or Fe^{2+} in combination with 2-oxoglutarate. Only a fraction of the maximal activity (15%) could be measured in the presence of 1 mM Ca^{2+}. Approximately 40 mM ascorbate and 0.5 mM Zn^{2+} were required for maximal hydroxylase activity (Figs. 2 and 3). Standard assays were carried out in buffer A at 30°C using optimal zinc and ascorbate concentrations. Under these conditions, the conversion of *trans*-4-coumaroyl-CoA to *trans*-caffeoyl-CoA was linear up to 90 min and up to 30 μg protein. *trans*-4-Coumaric acid was also hydroxylated efficiently to

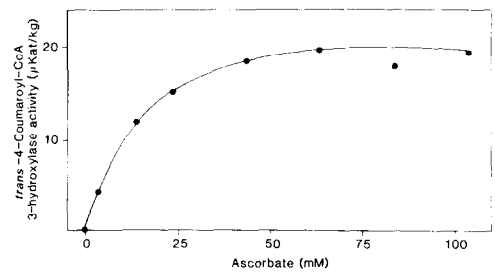

FIG. 2. Dependence of *trans*-4-coumaroyl-CoA 3-hydroxylase activity on ascorbate concentration. The incubations were carried out for 30 min at 30°C in 50 mM Mes/Mops buffer, pH 6.5, containing 0.5 mM zinc sulfate, 150 μM *trans*-4-[β-^{14}C]coumaroyl-CoA (275 Bq), and 30 μg of crude enzyme protein.

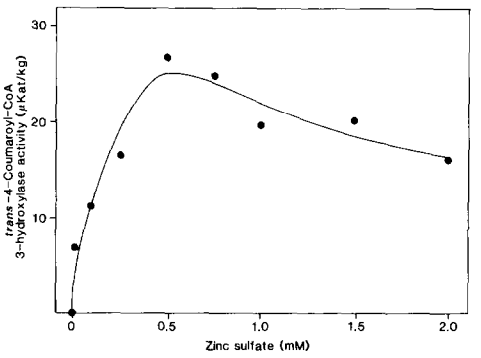

FIG. 3. Dependence of *trans*-4-coumaroyl-CoA 3-hydroxylase activity on Zn^{2+} concentration. The incubations were carried out as in Fig. 2 in the presence of 50 mM ascorbate.

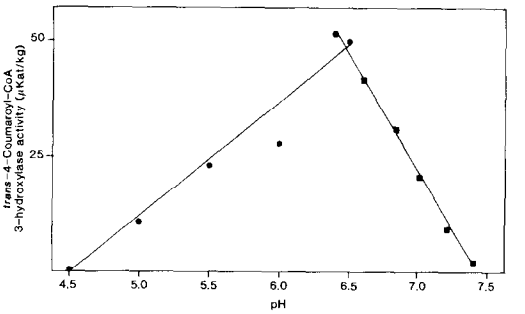

FIG. 5. Dependence of *trans*-4-coumaroyl-CoA 3-hydroxylase activity on buffer conditions. The pH was varied in either (●) 100 mM ammonium acetate (pH 4.5–6.5) or (■) 50 mM Mes/Mops (pH 6.4–7.5) buffer containing 50 mM ascorbate and 0.5 mM zinc sulfate.

trans-caffeic acid, whereas 4-coumaroylshikimate and 4-coumaroylquinate were not as readily accepted (25% of the maximal conversion rate with 4-coumaroyl-CoA as substrate). Cinnamic, ferulic, and 4-hydroxybenzoic acids were not accepted. No product other than the corresponding caffeoyl analogs or caffeic acid was formed. Kinetic analysis in crude extracts (Fig. 4) revealed that 4-coumaroyl-CoA (V_{max}/K_m = 3.16) and not 4-coumarate (V_{max}/K_m = 2.36) was the preferred substrate.

Dependence of the 3-Hydroxylase Activity on pH and Its Subcellular Localization

Probably due to the Zn^{2+} dependence of the hydroxylase activity, imidazole or phosphate buffers could not be used in the hydroxylase assay. In Mes/Mops or acetate buffers, however, maximal activity was observed at pH 6.5 with a sharp decline toward both alkaline and acidic conditions (Fig. 5). The activity was negligible at pH 7.5, although no precipitation of zinc salts occurred under these conditions. A similar dependency was measured in Pipes and Tris/HCl buffers with no activity detected at pH 7.5. The decrease in activity toward pH 7.5 was also not due to denaturation of the enzyme since full activity was measurable when the enzyme extract was kept at pH 7.5 and 4°C for 60 min prior to the assay at pH 6.5. The narrow, acidic pH optimum suggested that, *in vivo*, the hydroxylase is either located in the vacuole or subject to an unusual regulatory mechanism. After fractionation of vacuoles in a Ficoll gradient (21), the hydroxylase activity was recovered from the supernatant and from intact protoplasts sedimenting faster than the vacuoles, but no significant activity was found in the fraction containing vacuoles identified both microscopically and by its phosphatase activity. The hydroxylase therefore appears to occur *in vivo* as a soluble cytoplasmic enzyme.

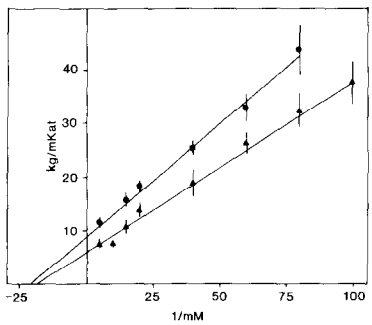

FIG. 4. Double-reciprocal plots of the initial velocities of *trans*-4-coumaric acid/*trans*-4-coumaroyl-CoA 3-hydroxylase activities with ascorbate and Zn^{2+} concentrations of 50 and 0.5 mM, respectively, and either (▲) *trans*-4-coumaroyl-CoA or (●) *trans*-4-coumaric acid as the variable substrate.

Effect of Elicitor Treatment on the 3-Hydroxylase Activity

Dark grown parsley cells contained a considerable hydroxylase activity (75

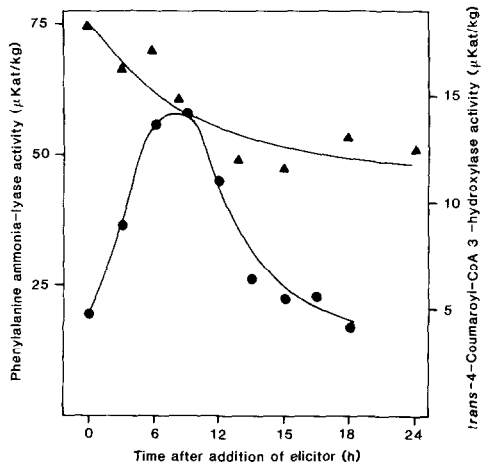

FIG. 6. Changes in (●) phenylalanine ammonia-lyase and (▲) *trans*-4-coumaroyl-CoA 3-hydroxylase activities in response to elicitor treatment of dark-grown, cultured parsley cells. At time 0, *Pmg* elicitor (4 mg) was added to the cell cultures (40 ml) and the cells were harvested after the time intervals indicated and deep frozen immediately. Extracts of the frozen cells were prepared and assayed for enzyme activity according to standard procedures.

μkat/kg) as determined *in vitro* by standard assays at pH 6.5, and the specific activity of extracts was not significantly changed by elicitor treatment of the cells. On the contrary, it decreased slightly over the time period of the experiment (Fig. 6). As a control, the well-known induction of PAL[2,3] activity by elicitor treatment of the cells was reconfirmed in these experiments (Fig. 6). Furthermore, the pH optimum of the hydroxylase activity did not change upon elicitor treatment of the cells. Our results suggest that the 3-hydroxylase must be inactive at the regular cytoplasmic pH of 7.4 (12, 13, 24) and requires a pH change for rapid activation rather than *de novo* enzyme synthesis.

Determination of Cellular pH

In ^{31}P NMR spectroscopy, the chemical shift of the inorganic phosphate resonance is sensitive to the pH of the microenvironment. This property has been exploited for the determination of the physiological pH range in live plant cells (12, 13, 24). Aerobic conditions during spectroscopy are necessary to keep the cytoplasmic pH as close as possible to that which is maintained under normal growth conditions. ^{31}P NMR spectroscopy of cultured parsley cells, which, for technical reasons, had been carried out previously under suboptimal conditions (13), was repeated under conditions of vigorous aeration. The relative signal intensities for the β phosphate in ADP and γ phosphate in ATP (both at −22 ppm) versus that of the β phosphate in ATP (−36 ppm) (Fig. 7) confirmed the aerobic condition of the cells.

As reported previously (13), elicitor treatment of the cells induced a rapid relative decrease in the cytoplasmic inorganic phosphate concentration and a relative in-

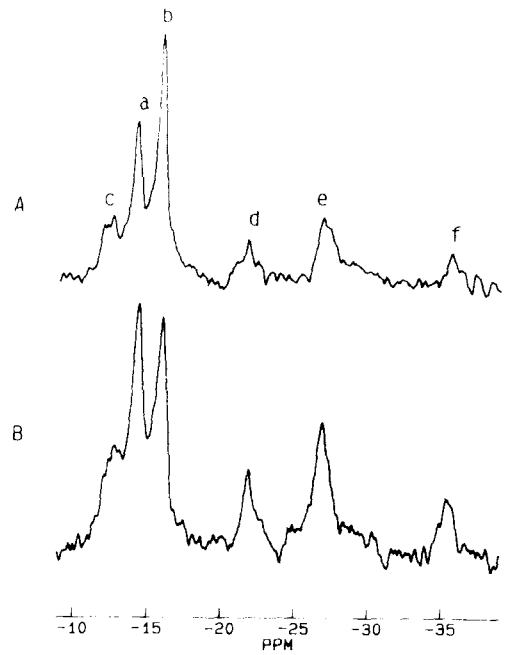

FIG. 7. ^{31}P NMR spectra of 6-day-old cultured parsley cells employing either (A) nonaerating conditions or (B) stirring the cell suspension in an air stream. The methylenediphosphonic acid signal at 1.925 Hz (0 ppm) served as the reference. The resonance signals are assigned as follows: a, cytoplasmic, inorganic phosphate; b, vacuolar, inorganic phosphate; c, sugar phosphates; d, e, f, signals for the γ, α, and β phosphates, respectively, of ATP. The α and β phosphate resonances of ADP also occur under the e and d signals, respectively.

crease in the vacuolar phosphate concentration (data not shown). Furthermore, the cytoplasmic pH of the cells decreased in a time-dependent manner by about 0.25 pH unit within approximately 55 min (Table I). The exact pH of the cytoplasm prior to elicitor treatment could be evaluated only by comparison with standard buffers, where minor changes in the ionic composition lead to relatively large shifts in resonance frequencies. Using the buffer composition suggested by Martin *et al.* (24), a pH of about 7.1 was estimated for the cytoplasm under aerobic control conditions. This appears unusually low (a pH of approximately 7.4 to 7.5 has been determined frequently (13, 24)) and may result from the choice of ionic composition of the reference buffers or from the transport of the cells over large distances before the measurements could be made.

TABLE I

TIME-DEPENDENT CHANGES IN THE ^{31}P RESONANCE FREQUENCY OF THE CYTOPLASMIC, INORGANIC PHOSPHATE IN ELICITOR-TREATED PARSLEY CELLS[a]

Time after addition of elicitor (min)	Chemical shift (ppm)	pH
Control	−14.00	7.07
8	−14.14	6.96
55	−14.32	6.84
80	−14.29	6.85

[a] The corresponding pH of the cytoplasm was determined from these frequencies by comparison with a reference buffer (50 mM potassium phosphate containing 100 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$). For control the cell suspension (10 ml) was stirred in the NMR tube and spectra were recorded several times over a time period of 2 h during which no change in the resonance frequency occurred. *Pmg* elicitor (2 mg) was subsequently added to the suspension and spectra were recorded frequently over the next 80 min.

DISCUSSION

The formation of hydroxycinnamic acid derivatives has been shown to be an integral part of the disease resistance expression of plant cells. These acids are responsible for the browning and the autofluorescence (26) of infection sites, at which the deposition of "lignin-like" materials has been frequently claimed to occur (5, 26, 28). More recently, caffeoyl and feruloyl esters have been implicated in playing a role in the defense response. Provided that H_2O_2 can be generated, these may become crosslinked by cell wall peroxidases (27). In parsley and other cultured plant cells, the browning of the cells in response to elicitor treatment is not due to lignification ((28, 29); unpublished results).

Nonspecific phenolases (monophenol monooxygenases), which have been reported to catalyze the formation of caffeic acid from 4-coumaric acid (14), or flavin-dependent monooxygenases (15) are, for various reasons (high K_m and pH optimum; negligible activity), unlikely to play a role in disease resistance expression. In this report we describe a novel, Zn^{2+}- and ascorbate-dependent hydroxylase which appears to prefer *trans*-4-coumaroyl-CoA over *trans*-4-coumaric acid as a substrate. The discovery of this enzyme activity makes the formation of caffeoyl-CoA from caffeoyl-shikimate, as speculated by Heller and Kühnl (16), rather unlikely. The fact that the hydroxylase is present in all cell cultures as yet examined (unpublished results) underlines its general role. This is further corroborated by the fact that a caffeoyl-CoA-specific 3-*O*-methyltransferase activity catalyzing the formation of feruloyl-CoA is also present in dark-cultured parsley cells and is induced by elicitor treatment of these cells (Pakusch *et al.*, manuscript in preparation).

The high concentration of ascorbate (40 mM) required for maximal 3-hydroxylase activity is reminiscent of the mammalian 4-hydroxyphenylpyruvate hydroxylase and the respective bacterial dioxygenase (30) and suggests that, *in vivo*, either another yet unknown reductant participates in the reaction or the ascorbate concentration represents a limiting factor for the enzyme activity. Similarly, the Zn^{2+} concentration determined *in vitro* for maximal activity appears to be unphysiologically

high. Since zinc is not a redox active metal, it is probably required for enzyme–substrate binding or hydroxyl transfer rather than for the redox process.

The 3-hydroxylase catalyzes the formation of 4-caffeoyl-CoA, 4-caffeate, and to a small degree 4-caffeoyl-shikimate/quinate under identical cofactor regimes. Several enzymes may, however, contribute to the total hydroxylating activity in the crude extracts, and it is not clear which of the substrates is converted best *in vivo*. Hydroxycinnamoyl-shikimate esters could not be recovered from the cells and the medium of dark-grown or elicitor-treated parsley cell suspension cultures (16). In addition, hydroxycinnamoyl-CoA:D-quinate hydroxycinnamoyltransferase activity could not be detected in parsley cell suspension cultures. This makes the shikimate and quinate esters of 4-coumarate unlikely substrates *in vivo*, and their hydroxylation *in vitro* was much less efficient (approx 25% of the maximal rate). The V_{max}/K_m ratios obtained *in vitro* with 4-coumaroyl-CoA and 4-coumaric acid as substrates might suggest that 4-coumaroyl-CoA is also *in vivo* a preferred substrate for the hydroxylase(s).

The hydroxylase present in the cytoplasm of dark-cultured parsley cells, would not be expected to be very active in the normal physiological pH range, nor is its *de novo* synthesis increased upon elicitor treatment of the cells. The regulation of metabolic processes has, however, in several instances been ascribed to changes in intracellular pH (31, 32). The elicitor-induced pH changes revealed in parsley cells by ^{31}P NMR spectroscopy would significantly modulate the hydroxylase activity. The steep increase in the hydroxylase activity in the pH range from 7.4 to 6.5 suggests that the hydroxylation of 4-coumaroyl-CoA to caffeoyl-CoA could be switched on very rapidly by appropriate *in vivo* pH changes. Together with phenylalanine ammonia-lyase and 4-coumarate:CoA ligase (33) activities, caffeoyl-CoA (and feruloyl-CoA) can be produced very soon after elicitor addition. It is interesting to note that these three enzymes, in contrast to the enzymes specific for phytoalexin synthesis in parsley (34), show high background activities. This points to their coordinated regulation in one biochemical pathway. Rapid availability of the abovementioned CoA esters is most probably essential for the first line of disease resistance expression, where time for *de novo* enzyme synthesis is not available.

Since neither feruloyl-CoA nor caffeoyl-CoA are substrates for phytoalexin synthesis in parsley (35), the 3-hydroxylation of 4-coumaroyl-CoA must represent a branch in the induced phenylpropanoid pathways, diverting the use of 4-coumaroyl-CoA from phytoalexin biosynthesis to the synthesis of cinnamic esters and related polymers (5, 26–28). The fact that furanocoumarin phytoalexins, which require the *ortho*-hydroxylation of 4-coumaric acid, are produced later in response to elicitor treatment demonstrates that the elicitor triggers the expression of a temporal pattern in the phenylpropanoid biosynthesis of parsley cells. This pattern may be expressed by either the relative biosynthetic capacity for coumaroyl-CoA versus caffeoyl-CoA or, most probably, by the transient drop in pH and caffeoyl-CoA formation.

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