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WOUNDING-INDUCED CINNAMIC ACID HYDROXYLASE IN JERUSALEM ARTICHOKE TUBER

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Abstract—Cinnamic acid hydroxylase (CAH), which catalyses transformation of *trans*-cinnamic acid into *p*-hydroxycinnamic acid, is a multi-enzyme system localized on the endoplasmic reticulum. Electrons are transferred from NADPH, the preferential electron donor for the system, to cytochrome P-450 via NADPH–cytochrome P-450 reductase, an enzyme regulated by the NADPH–$NADP^+$ ratio. The induction and subsequent changes of CAH activity during ageing are accounted for by the variations in reductase and cytochrome P-450 content. The content of cytochrome b_5, already present in the dormant tuber, is markedly enhanced by wounding; its participation in electron transport from NADH to the hydroxylase is discussed.

INTRODUCTION

Slicing and aeration of quiescent storage tissues induces a rapid metabolic activation and a development of the endomembranous system in the wounded tissues. The observed dedifferentiation might be the result of gene derepression, leading to enhanced RNA synthesis, polyribosome formation, structural and enzymatic protein production, lipid and sterol biosynthesis, degradation of storage substances [1].

Cinnamic acid 4-hydroxylase (CAH), a key enzyme in phenylpropanoid biosynthesis [2], is located on the endoplasmic reticulum (this report), a subcellular fraction differentiating early among those organelles developing during ageing of storage tissues [3–5]. The enzyme activity was induced by slicing in Jerusalem artichoke tuber tissues [2] and identified as a mixed function oxygenase involving cytochrome P-450 [6] for the hydroxylation of cinnamic acid into *p*-hydroxycinnamic acid. This paper reports further investigations on the properties of CAH and the effects of wounding on the development of this multienzyme system in disks of Jerusalem artichoke (JA) tuber tissues.

RESULTS

The product of the reaction catalysed by CAH was identified as *p*-coumaric acid by TLC in Et_2O–hexane (1:1). Further confirmation was gained from MS studies after methylation of the reaction product and of authentic *p*-coumarate followed by TLC cochromatography in EtOAc–cyclohexane (2:3). Czichi and Kindl [7] reported the formation of *o*-coumaric acid by potato microsomes. No *o*-hydroxylase could be detected in JA microsomes.

Maximum CAH activity was associated with a 100000 *g* pellet, whereas the supernatant was devoid of activity. A 10000 *g* pellet exhibited only 5% of the total CAH activity. Resolution of the 1000 *g* supernatant into its constitutive membranes was partially achieved on a sucrose density gradient (Fig. 1). Maximum NADPH–cytochrome *c* (cyt *c*) reductase activity, a marker enzyme of endoplasmic reticulum (ER), showed good coincidence with maximum CAH activity. Succinate–cyt *c* reductase, a marker enzyme of mitochondria, was however present in some fractions containing CAH activity. This light mitochondrial fraction might represent either fragments or neosynthesized mitochondria.

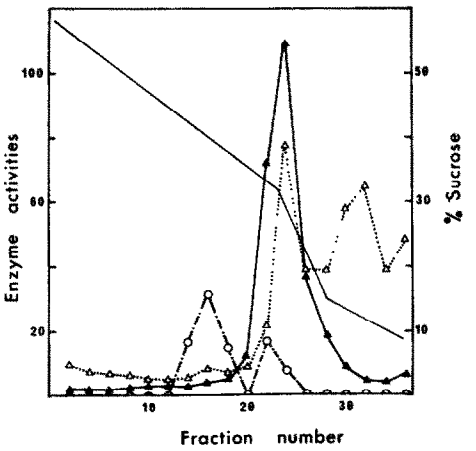

Fig. 1. Distribution of marker enzymes on an isopycnic sucrose gradient O- - - -O succinate–cyt *c* reductase (μmol cyt *c* reduced/min/fraction); Δ.....Δ NADPH–cyt *c* reductase (μmol cyt *c* reduced/min/fraction); ▲——▲ cinnamic acid 4-hydroxylase (pmol *p*-coumaric acid formed/min/fraction); ——— sucrose conc.

The apparent K_m for cinnamic acid, calculated from the reciprocal plot of initial CAH activity as a function of substrate conc was 5×10^{-6} M. *p*-Coumaric acid, the product of reaction, at a conc from 50 to 200 μM had no detectable inhibitory effect on CAH activity.

O_2 is required for CAH activity: disks of tuber tissues

placed in a N_2 atmosphere and incubated with phenylalanine-[^{14}C] accumulated cinnamic acid-[^{14}C] [2]. When microsomes were incubated in a high conc of carbon monoxide (90%) CAH activity was inhibited by 95%. With a gas mixture of 10% CO, 10% O_2 and 80% N_2, CAH activity was 54% of the control incubated in air, when the reaction occurred in darkness, while only a 24% inhibition was measured when the incubation was carried out in white light.

The enzyme activity was optimal in a pH range of 6.7 to 7 and half-maximal at pH 6 and 7.8.

Table 1. Cofactor requirement for the CAH activity

Cofactors added	Conc (mM)	CAH activity: nmol *p*-coumarate formed/min/microsomes from 550 disks
No cofactor	—	0
Ascorbic acid	0.3	0
NADH	0.5	0.9
NADPH	0.01	22.3
NADPH*	0.01	16.6
NADPH	0.5	42.4
NADPH	0.01	33.7
+NADH	0.05	
NADPH	0.01	35.5
+NADH	1.0	
NADPH	0.5	56.5
+NADH	0.5	

Disks were aged for 16 hr. Microsomes were incubated at 25° for 10 min. All nucleotides were supplied along with a regenerating system (alcohol dehydrogenase and EtOH for NADH, G-6-P dehydrogenase and G-6-P for NADPH). * In this test NADPH was supplied without regenerating system.

Table 1 indicates the cofactor requirement for the hydroxylation of cinnamic acid by microsomes of aged tuber tissues. Without external cofactor, microsomes could not catalyse the conversion of cinnamic acid to *p*-coumaric acid. Ascorbic acid did not support CAH activity. NADH allowed only a low rate of hydroxylation at a concentration where NADPH permitted maximal CAH activity. Although NADH alone could not supply CAH with electrons, in the presence of NADPH it exerted a synergistic effect on the hydroxylation reaction: at a non saturating conc of NADPH (0.01 mM), NADH (0.5 mM) resulted in 51% stimulation of CAH; when NADPH was present at a saturating level (0.5 mM), NADH (0.5 mM) still produced a 33% stimulation of CAH activity. Increasing the NADH conc did not enhance greatly its stimulatory effect.

Table 2. Effect of the ratio NADPH/$NADP^+$ on CAH activity

NADPH/ $NADP^+$	Conc of NADPH (μM)	Conc of $NADP^+$ (μM)	% inhibition of CAH activity
0.125	25	200	74
0.25	25	100	55
	50	200	59
0.50	50	100	45
	100	200	43
1.00	100	100	26

Microsomes were incubated at 25° for 5 min.

An apparent K_m of 1.3×10^{-5} M for NADPH was calculated from the effect of NADPH conc on the initial velocity of the hydroxylation reaction. $NADP^+$ acted as a competitive inhibitor ($K_i = 2.4 \times 10^{-5}$ M) of the reaction, presumably at the NADPH–cyt P-450 reductase level, because the same inhibition was observed when measuring the cyt *c* reductase activity of cyt P-450 reductase. As shown in Table 2, CAH activity was not only dependent on NADPH conc, but also on the ratio NADPH:$NADP^+$.

Table 3 shows that in the presence of $NADP^+$, NADH could support CAH activity, an effect stimulated (×5) by ATP. An ATP-activated microsomal transhydrogenase was described in *Echinocystis* [8]. Hydroxylation of cinnamic acid could also be driven by $NADP^+$ plus glucose-6-phosphate (G-6-P), indicating the presence of a membrane-bound G-6-P dehydrogenase located on JA tuber microsomes.

Table 3. Capacities of microsomes to produce NADPH

Cofactors added	Conc (mM)	CAH activity. nmol *p*-coumarate formed/min/microsomes from 550 disks
NADPH	0.75	59.8
$NADP^+$	0.75	0
$NADP^+$	0.75	
+NADH	1.5	2.1
$NADP^+$	0.75	
+NADH	1.5	10.3
+ATP	1.5	
$NADP^+$	0.75	
+ATP	1.5	5.1
NADH	1.5	
+ATP	1.5	5.1
$NADP^+$	0.75	
+G-6-P	1.5	36.1

Disks were aged for 16 hr. Microsomes were incubated at 85° for 20 min.

The effect of wounding on CAH activity in disks of JA tuber is shown on Fig. 2. The dormant tuber had no detectable CAH activity *in vitro*. In response to slicing, after a lag phase of 1 hr, CAH activity increased rapidly and reached a maximum after 16 hr. Then CAH activity decreased and stabilized at a low level 48 hr after cutting. This pattern of development for CAH activity was obtained with freshly harvested tubers. Prolonged storage of the tubers resulted in an increase of the time needed to reach maximal CAH activity and a decrease of the absolute activity.

Evolution, after slicing, of cyt P-450, responsible for binding of cinnamic acid [6], and for binding, activation and insertion of O_2 into the substrate, is represented on Fig. 2. Present at a low level in dormant tuber tissues, the total amount of cyt P-450 increased rapidly in response to excision and reached a peak 16 hr after cutting. Thereafter the conc of cyt P-450 decreased slightly, but it remained at a relatively high level.

Reduction of cyt P-450 by NADPH, the most effective electron donor for CAH activity as shown in Table

1, proceeds *via* the flavoenzyme NADPH–cyt P-450 oxidoreductase. The enzyme activity was measured using cyt *c* as substrate. NADPH–cyt *c* reductase increased markedly in the microsomal fraction from a low rate in the dormant tissues to a high activity 16 hr after wounding and dropped drastically between 16 and 24 hr, reaching the initial activity after 48 hr (Fig. 2).

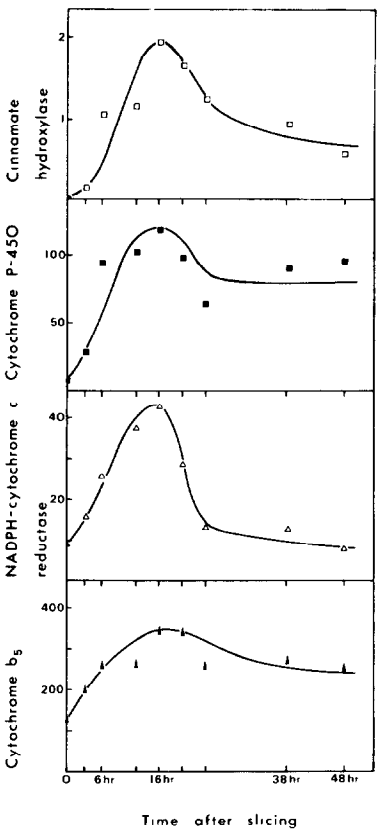

Fig. 2. Effect of wounding on components of the microsomal multienzyme system hydroxylating cinnamic acid: □——□ CAH (nmol *p*-coumaric acid formed/min/mg protein; ■——■ cyt P-450 (pmol/mg protein); △——△ NADPH-cyt *c* reductase (nmol cyt *c* reduced/min/mg protein) and ▲——▲ cyt b_5 (pmol/mg protein).

Results reported in Table 1 show that under certain conditions NADH could participate in cyt P-450 reduction. It has been proposed [9] that electrons could be transferred from NADH to cyt P-450 through a specific flavoprotein and cyt b_5. Figure 2 shows that cyt b_5, already present in the microsomes of the dormant tuber, increased after slicing during 16 hr and thereafter decreased slowly again.

DISCUSSION

The occurrence of mixed function oxygenases in plants is now well documented. This type of enzyme takes part in gibberellin biosynthesis [10,11], terpene [12] and phenolic metabolism. The involvement of cyt P-450 in cinnamate hydroxylation was first suggested by Russell [13] on the basis of the light reversal of CO-poisoning of the reaction. In a previous paper [6] we reported the presence of cyt P-450 in microsomes of JA tuber tissues and the involvement of this cyt in CAH activity based on the type I difference spectra of the enzyme–substrate complex [14]. Such spectra were observed recently with potato tuber microsomes by Rich *et al.* [15]. Binding of cinnamate and aniline to plant cyt P-450 was also inferred from EPR studies [16]. Further confirmation was given by an action spectrum of light reversal of the CO inhibition of the reaction in sorghum seedling microsomes [17]. We report herein further studies on the enzyme location, the availability and route of reducing equivalents and the induction of the multienzyme complex in response to wounding.

In contrast to the finding by Camm and Towers [18] that CAH in potato tuber was soluble in the quiescent and microsomal in the ageing tissue, we found no CAH activity at the time of slicing and the enzyme was always membrane-bound. With few exceptions, the monooxygenases of eukaryotes are embedded in the ER. This is true also for the CAH of JA tuber as evidenced by continuous and discontinuous (not shown) gradients. In the latter, where membrane conc enables cyt determination, cyt P-450 and CAH were in very good coincidence with NADPH–cyt *c* reductase, the marker for ER. In contrast to the observation of Tanaka *et al.* [19] with sweet potato, addition of Mg^{2+} to the gradient did not dissociate CAH and NADPH–cyt *c* reductase activity.

As indicated in Table 1, NADPH was the preferential electron donor for CAH activity. If G-6-P was supplied, $NADP^+$ was reduced by a G-6-P dehydrogenase, bound to the same membrane fraction. Its *in vivo* participation in cinnamate hydroxylation and role in regulation is uncertain. It is admitted that NADPH–cyt *c* reductase is identical to cyt P-450 reductase, the flavoenzyme mediating the electron transfer from NADPH to cyt P-450. We observed that $NADP^+$ produced the same type of competitive inhibition of both activities, the latter (cyt P-450 reductase) being measured by CAH activity ($K_i = 2.4 \times 10^{-5}$). Table 2 shows that CAH activity was dependent upon the NADPH/$NADP^+$ ratio and not $NADP^+$ conc. A preliminary study showed that the NADPH/$NADP^+$ ratio varied in ageing tissues (unpublished). The effect of $NADP^+$ explains why, at non-saturating conc, NADPH was much more effective if supplied along with a generating system (Table 1): not only was the conc of NADPH maintained constant, but $NADP^+$ was removed, which otherwise would have inhibited the reductase. NADH supported appreciable hydroxylation only if supplied with the other nucleotide. Electrons might be transferred from NADH to cyt P-450 either directly *via* cyt b_5 [20] or *via* NADPH after transhydrogenation. An ATP-activated transhydrogenase was described by Hasson and West [8]. We were unable to measure direct transhydrogenation using West's conditions, but NADH supplied together with $NADP^+$ supported cinnamate hydroxylation and ATP enhanced the enzyme activity. It was argued [21] that transhydrogenation cannot explain the role of NADH because increased $NADP^+$ conc inhibited CAH activity. We observed the same effect but, since $NADP^+$ can be at the same time substrate for the transhydrogenase and inhibitor toward cyt P-450 reductase, a definitive conclusion cannot be drawn at the present time. It is however obvious that transhydrogenation cannot account for an important part of CAH activity. Table 1 shows that

NADH, a poor electron donor for CAH activity, exerts synergistic effects in the presence of NADPH on the hydroxylation rate of cinnamate, even in the case of a saturating level of NADPH. This differs from the effect observed in swede root microsomes [21], where NADH had little effect on the V_{max} of the reaction. Our results are best reconciled with the hypothesis of Staud *et al.* [22], that the NADH–cyt b_5 pathway would prevent the liberation of H_2O_2 within the microsomal membrane rather than directly participate in cyt P-450 reduction [20,23]. Polarographic measurements of O_2 uptake in JA microsomes showed a strong background oxidation of NADH, a possible result of the high degree of auto-oxidabilities of cyt b_5 in our material. This might explain the relatively high conc of NADH required in the experiments of Table 1.

The content of cyt P-450 was, in the dormant tuber, at a very low level, barely detectable, but increased dramatically upon slicing. Considering that the cyt, which binds cinnamate and O_2, is the enzyme *sensu stricto*, the observed increase could be due to *de novo* synthesis if one assumes that modulation of degradation is not the reason of the changes in cyt P-450 content. The use of cycloheximide (20 μM), although controversial [24], resulted in the total inhibition of cyt P-450, reductase and CAH. Although cinnamate hydroxylation certainly does not account for all the measured cyt P-450 we assume that it constitutes the greatest part of it in the present case, i.e. a tissue engaged in rapid dedifferentiation and lignification. Our results (Fig. 2) show a good correlation between CAH activity and cyt P-450 content during the induction phase and a good agreement between CAH and cyt P-450 reductase activities after 16 hr of ageing: CAH activity would be at any one moment the resultant of the relative amount/activity of its constituents. However, if multiple cyt P-450s exist, there is a possibility that wounding would induce different cyt P-450s with different time courses and therefore cyt $P\text{-}450_{cin}$ would not be so well correlated to variations in total cyt P-450 estimated spectrophotometrically. It was shown in parsley cell suspension cultures [25] that the lipid fraction generally associated to monooxygenases does not intervene in the regulation of CAH activity by light. The appearance of new hydroxylase activities (e.g. lipid or sterol monooxygenase) could explain the fact that cyt P-450 was still at a relatively high level after 48 hr, although CAH activity was very low. In contrast to cyt P-450, the second hemoprotein present in the microsomes, cyt b_5, was detected in significant amounts in the dormant tuber. Wounding however resulted in a marked increase followed after 16 hr by a slowly declining plateau. So far, our results suggest that cyt b_5 does not directly take part in the monooxygenase electron transport. The resolution in its constitutive elements and subsequent reconstitution of the hydroxylating system should bring more information on the enzyme mechanism itself. The problem arises also of the specificity of the cyt P-450 induced by wounding: Could it hydroxylate other substrates? Is there a plurality of specific cyt P-450 or a few large spectrum hydroxylases?

EXPERIMENTAL

Disks (0.5 mm × 10 mm) of Jerusalem artichoke (*Helianthus tuberosus*, var. blanc commun) tubers were vigorously shaken in 100 ml conical flasks in H_2O at 25°. After different times of ageing, disks were homogenized in 2.5 vol NaPi buffer, pH 7.4, in the presence of 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.4% bovine serum albumin, 0.6 M mannitol and 10% (wt/fr. wt) polyclar AT. Crude extract was filtered through cheesecloth and centrifuged at 10000 *g* for 15 min. The supernatant obtained was centrifuged at 100000 *g* for 1 hr, the microsomal pellet formed was suspended in 0.1 M NaPi buffer, pH 7.4, containing 10 mM 2-mercaptoethanol. When indicated, microsomes were washed in a sufficient vol of resuspension medium, resedimented by centrifugation at 100000 *g* for 1 hr and resuspended in the same medium. The sucrose of the density gradients was dissolved in 0.1 M NaPi buffer, pH 7.4, containing 1 mM EDTA. Gradients consisted of 5 ml 60% sucrose, 20 ml of a linear gradient from 60–30% sucrose and 5 ml of 15% sucrose. 8 ml of a 1000 *g* supernatant were layered onto the gradient and centrifuged in a Spinco rotor SW 27 at 20000 rpm for 4 hr at 4°. 1 ml fractions were collected and assayed for CAH activity, NADPH–cyt *c* reductase and succinate–cyt *c* reductase [26]. Sucrose conc were determined by refractometry. CAH activity was measured after the radiochemical method of ref. [13], modified for the vol of incubation (0.2 ml). The microsomes were incubated for 20 min at 25° and the reaction was stopped by 20 μl 4 M HCl. Carrier cinnamic acid and *p*-coumaric acid (0.1 mg) were added to the reaction mixture and the ppt was sedimented. To separate the residual substrate and the product of the reaction, an aliquot of the reaction mixture was spotted on a fluorescent Si gel plate and chromatographed in C_6H_6–HOAc–H_2O (6.7.3, upper phase). The spot of *p*-coumarate, detected by its fluorescence in UV light, was directly scraped into a scintillation vial and eluted with the scintillation soln (toluene–PPO–POPOP). The content of cyt P-450 was determined as described in ref. [27] using an E of 91 mM^{-1} cm^{-1}. Cyt b_5 was measured after ref. [27] using an E of 161 mM^{-1} cm^{-1} between 425 and 410 nm for dithionite reduced microsomes. Although widely used [22,28] this method can lead to overestimation of cyt b_5 by titrating any high potential cyt that might be present in the preparation. Somewhat lower values were obtained with NADH reduced microsomes but the time course of cyt b_5 evolution was unchanged. Protein was determined according to ref. [29].

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