

PHYTOCHROME-MEDIATED REGULATION OF A MONOOXYGENASE HYDROXYLATING CINNAMIC ACID IN ETIOLATED PEA SEEDLINGS

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Abstract—Cinnamic acid is hydroxylated by the mixed-function oxidase *trans*-cinnamic acid 4-hydroxylase (CA4H). The hydroxylation reaction involves the transfer of electrons from reduced pyridine nucleotides via the enzyme NADPH cytochrome P-450 reductase to the terminal oxidase cytochrome P-450. This multi-enzyme complex is localized in the microsomal fraction. Isopycnic and velocity gradient centrifugation suggest that in the apical bud of etiolated pea seedlings this complex is restricted to the endoplasmic reticulum membranes. CA4H activity which develops in dark germinating pea seedlings was found to be stimulated by light, an effect mediated by phytochrome. CA4H and NADPH cytochrome *c* reductase activities, cytochromes P-450 and *b* 5 contents were measured in seedlings submitted to either short pulses of red and far-red light, or to continuous far-red or blue irradiation. The results are discussed in terms of a specific effect of phytochrome on the different parts of the multi-enzyme complex.

INTRODUCTION

The involvement of light in the regulation of the biosynthesis of flavonoids and their precursors, the phenylpropanoids, is well documented [1]. The first enzymes of this biosynthetic pathway, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), cinnamic acid 4-hydroxylase (CA4H, EC 1.14.14...) and *p*-coumaryl CoA ligase show similar time-courses under white light illumination in buckwheat [2] and parsley cell suspension cultures [3, 4]. In most higher plants the light-regulation of PAL is phytochrome-mediated [5–7] although a blue absorbing pigment clearly intervenes in certain species [8, 9]. In the pea seedlings both phytochrome and the blue-absorbing pigment are involved in flavonoid biosynthesis [10] and PAL regulation [9]. CA4H was first described in this material [11, 12] and the enzyme has gained considerable interest since the recognition of its monooxygenase nature. Its activity requires the terminal oxidase cyt P-450 which binds the substrates cinnamate and oxygen [13–16], a flavoprotein transferring electrons from NADPH to the cyt [16, 17], and a lipid factor [18].

In this paper we report on the subcellular localization of the constituents of CA4H and their phytochrome-dependent evolution in apical buds of etiolated pea seedlings. The possible mechanisms of phytochrome-mediated CA4H activity changes are discussed in terms of a specific action on the different elements of this multi-enzyme complex.

RESULTS AND DISCUSSION

The sub-cellular localization of CA4H in the apical bud of the pea seedling was ascertained by the resolution of the 1000 *g* supernatant by continuous sucrose density gradient centrifugation. All CA4H activity was associated with a light fraction (1.12 g/cm³) and was generally in perfect coincidence with NADPH-cyt *c* reductase, a marker for the endoplasmic reticulum (ER) [19]. In

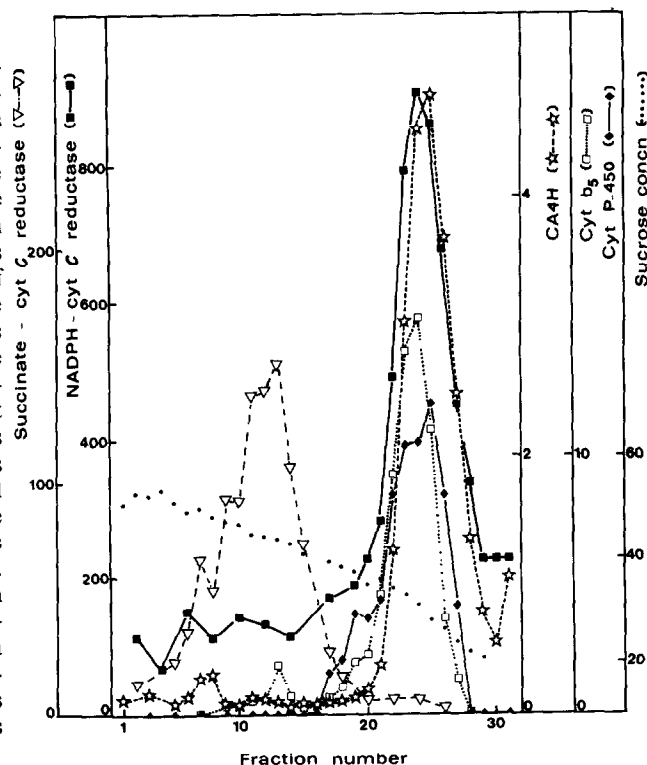


Fig. 1. Localization of CA4H, cyt P-450 and cyt *b* 5 from a 1000 *g* supernatant of apical buds of pea seedlings on a sucrose density gradient after isopycnic centrifugation. ★—★ CA4H (pkat *p*-coumarate formed/ml fraction); ◆—◆ Cyt P-450 ($\Delta A_{(450\text{nm}-490\text{nm})} \times 10^3/\text{ml}$ fraction); □—□ Cyt *b*₅ ($\Delta A_{(424\text{nm}-410\text{nm})} \times 10^3/\text{ml}$ fraction); ▽—▽ Succinate-cyt *c* reductase (pkat cyt *c* reduced/ml fraction); ■—■ NADPH-cyt *c* reductase (pkat cyt *c* reduced/ml fraction); Sucrose concn (% wt/vol).

some runs however (Fig. 1), CA4H banded at a slightly lower buoyant density than the reductase (1.127 g/cm^3). The reason for this discrepancy is not yet known. The mitochondrial fraction, as characterized by succinate-cyt *c* reductase, was devoid of CA4H and NADPH cyt *c* reductase activities. We did not observe, in the pea seedlings, CA4H in fractions possessing catalase activity as was found in the germinating castor bean endosperm [20]. Although the highest concn of cyt P-450 and cyt *b* 5 coincided with the highest CA4H and NADPH cyt *c* reductase activities, their distribution over the gradient consistently presented a shoulder ($1.135\text{--}1.14 \text{ g/cm}^3$) which could not yet be correlated with specific membrane fraction. Recently, a monooxygenase involved in indole alkaloid synthesis in *Catharanthus roseus* seeds was found in a $20000g$ pellet, which banded at 1.13 g/cm^3 when subjected to discontinuous sucrose gradient centrifugation; this fraction was assigned as provacuolar vesicles [21].

The possibility remained that the ER-enriched fraction might be contaminated by light fragments of other organelles, as plasmalemma and disrupted plastids.

In the maize coleoptile which exhibits good CA4H activity the ER can be well separated from the plasmalemma (i.e. β glucan synthetase with low affinity for UDPG) [22]. In this material CA4H activity coincided well with the ER and none was associated with the plasmamembranes. The fact that some UDP-galactose galactosyl transferase activity (not shown), a known marker enzyme of the plastid membrane [23], banded at the same density as the ER showed that the latter was contaminated by some plastid material. Since CA4H activity has been reported in the chloroplast of a green algae [24], we used the velocity sedimentation method according to ref. [25] to separate the ER from the intact plastids of de-etiolated (120 hr white light) pea seedlings. As shown in Fig. 2, there is no significant CA4H activity in the plastid-enriched fraction. Although this cannot exclude that in etiolated or FR-de-etiolated seedlings some CA4H activity might be associated with etioplasts, our data strongly suggest that in our material the CA4H complex is restricted to the ER membranes.

There is now ample evidence that CA4H is a cyt P-450 monooxygenase. The pea seedling enzyme exhibited

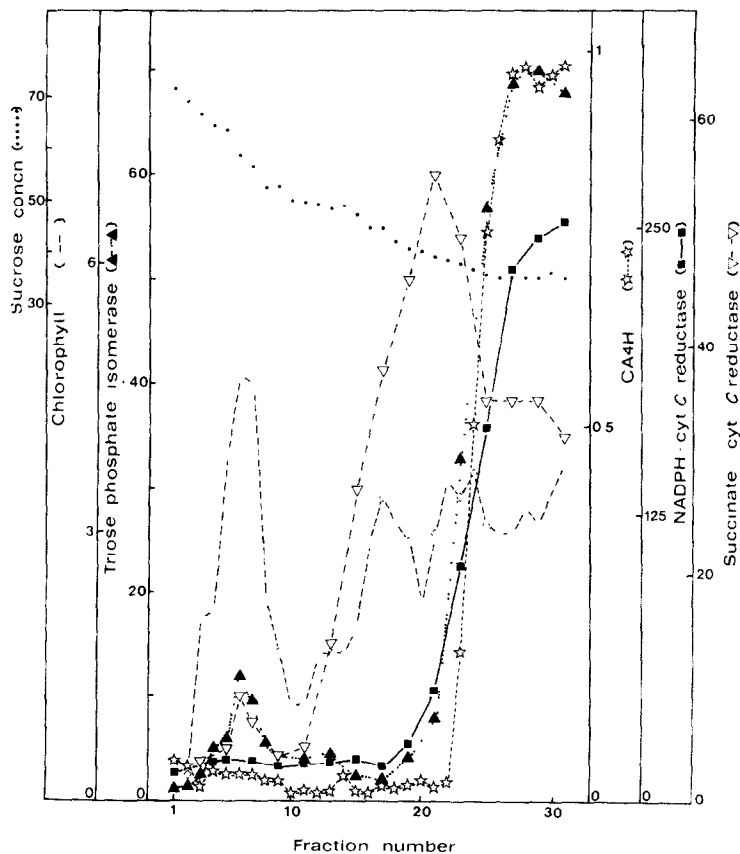


Fig. 2. Distribution of CA4H, marker enzymes of ER, mitochondria and intact plastids, and chlorophyll from a crude homogenate of green pea seedling apical buds on a sucrose density gradient after velocity centrifugation. ☆—☆ CA4H (pkat *p*-coumarate formed/ml fraction); ▽—▽ Succinate-cyt *c* reductase (pkat cyt *c* reduced/ml fraction); ■—■ NADPH-cyt *c* reductase (pkat cyt *c* reduced/ml fraction); ▲—▲ Triose phosphate isomerase (pkat NADH oxidized/ml/fraction); ◇—◇ Chlorophyll (mg/ml fraction); Sucrose concn (% wt/vol.).

Table 1. Photoreversibility by FR-light of the R-light induced increase of CA4H and NADPH-cyt *c* reductase activities, and of cyt P-450 and cyt *b* 5 concn

Data expression	Light treatment	CA4H activity (pkatals)	NADPH-cyt <i>c</i> reductase activity (pkatals)	Cyt P-450 concn (pmol)	Cyt <i>b</i> 5 concn (pmol)
(a) per bud		(1)	(2)	(3)	(4)
	0	0.07	41	2.66	8.53
	10 min FR	0.12	40	5.61	14.34
	10 min FR + 5 min R	0.22	66	7.14	17.97
	5 min R + 10 min FR	0.13	49	5.72	15.08
(b) per g fr. wt		(5)	(6)	(7)	(8)
	0	8.82	4220	516	1392
	10 min FR	11.38	3980	558	1413
	10 min FR + 5 min R	17.1	5230	562	1430
	5 min R + 10 min FR	11.23	4530	517	1362

After the light treatment, the seedlings were returned to darkness for 18 hr prior to extraction. Values are means of 6 independent experiments (5 for reductase). In the Student and Fisher test, the R effect and R-FR photoreversibility are significant at the 99 % confidence level in sets (1), (2), (6), at the 95 % confidence level in sets (3), (4), (5), and not significant in sets 7 and 8. In set 6 however the reversion is significant only at the 70 % level.

properties very similar to those we found for the Jerusalem artichoke enzyme [17] and others described for the potato tuber [16], the swede root [26] and the sweet potato [27] enzymes. Pea CA4H was inhibited by anaerobiosis and CO, the latter inhibition being reversed by light. Maximum enzyme activity was observed at pH 7.2, the optimum temperature was 30° and the reaction was linear over 20 min at 25°. *o*-Coumaric acid was not formed under our assay conditions and *p*-coumarate, the only product detected, did not inhibit the reaction, even at non saturating cinnamate concentration. The kinetic behaviour of the pea enzyme differs from those described so far and will be discussed in a forthcoming paper.

It is well known [2, 3, 12] that light stimulates CA4H, along with several other enzyme activities, during seedling de-etiolation. This is not the case, however, in aging potato tuber discs [28] where PAL but not CA4H is photo-stimulated. In the pea seedling apical buds, both blue and red light are effective in promoting CA4H activity: the involvement of phytochrome in the red light regulation of the enzyme is demonstrated by the R-FR photo-reversion observed on CA4H activity (Table 1). The data differ, whether expressed per apical bud (Table 1a) or per g fr. wt which parallels microsomal protein content (Table 1b). If expressed per g fr. wt, the cyt content no longer seems to be affected by P_{fr} whereas CA4H and the reductase are still stimulated. One could assume that P_{fr} exerts a two-sided effect: (i) a general stimulation of membrane synthesis resulting in cyt P-450 and cyt *b* 5 concn enhanced per apical bud but unchanged per fr. wt, (ii) a more specific effect on CA4H and reductase activities stimulated whichever parameter they are referred to. From ref. [29], one can estimate that FR establishes in our material a photostationary state with a few percent of total phytochrome in the active P_{fr} form. After red pulses, P_{fr} was shown [30] to decay in a microsomal fraction from pea shoots with a half-life of about 60 min. It is apparent (Table 1a) that the low P_{fr} level produced by 10 min FR suffice to stimulate CA4H (72 %), cyt

P-450 (110 %) and cyt *b* 5 (68 %) but is ineffective in promoting any reductase activity change. The specific activity* of CA4H (Table 1b) increases by 30 % and a slight stimulation of cyt P-450 concn is observed. This could be due, the existence of multiple cyt P-450 forms being likely [16], to the induction of a particular cyt P-450_{cinnamate} species. When R light is given following FR irradiation ($P_{fr} \approx 80$ % of total P), NADPH cyt *c* reductase is stimulated and CA4H activity further enhanced. FR following R almost completely abolishes the R triggered stimulation.

Data in Table 2 show that it is not the high level of, but the time during which, a certain amount of P_{fr} is maintained that is of importance for reductase stimulation. Thus, P_{fr} seems to exert a selective effect on the different constituents of CA4H activity: CA4H and cyt concn are enhanced by low P_{fr} levels established by a short period of time whereas NADPH cyt *c* reductase is stimulated only by longer irradiation times. A further clarification of this point will necessitate dose-response curves and P_{fr} level manipulation by varying the wavelength of actinic light.

Under continuous FR light CA4H and NADPH cyt *c* reductase activities increased after a short lag phase (≈ 45 min) reaching a maximum after 30 hr and then decreasing (Fig. 3). In the etiolated seedlings these activities remained constant for the period considered. Maximal stimulation for CA4H and reductase was 3.5 and 3 fold, respectively. The time-course of cyt P-450 and cyt *b* 5 (not shown) concn evolution was

Table 2. Effect of increasing FR irradiation on the stimulation of CA4H and NADPH cyt *c* reductase activities

Light treatment	CA4H activity (pkatals g ⁻¹ fr. wt)	NADPH-cyt <i>c</i> reductase (pkatals g ⁻¹ fr. wt)
0	10.9	3730
10 min FR	14	3520
20 min FR	14	4340
30 min FR	15.7	4560

After the light treatment, the seedlings were returned to darkness for 18 hr prior to extraction. The slight increase in reductase activity after 10 min FR was consistently observed.

* Specific activity is used in a broad sense of enzyme activity per microsomal protein.

similar, maximal stimulation after 30 hr FR being 2 for both cyt. Here again, cyt *c* reductase and CA4H activities are more greatly stimulated than the average microsomal protein, whereas cyt P-450 and cyt *b* 5 concn parallels the protein content.

PAL activity (not shown) was also measured and as in many cases [2-4, 31, 32] exhibited activity changes parallel to those of CA4H. Return of the seedlings to darkness following 6 hr FR irradiation led to an equal loss in both enzyme activities. There is evidence for [33, 34] and against [35] phytochrome mediated *de novo* synthesis of PAL. Our data show that when CA4H is stimulated, the cyt P-450 titer increases no more than the average microsomal protein content. One could therefore assume that P_{fr} triggers an activation of CA4H, possibly by increasing the reductase activity. This could be due either to an increased reductase activity or to facilitated electron transfer by phytochrome induced topological or energetic changes in the state of the membrane. It is very likely that multiple cyt P-450 species occur in plants as in mammals and hence, that cyt P-450_{cinnamate} would be only a part of the spectrophotometrically detected cyt P-450. The possibility then exists that of all the different putative cyt P-450, only cyt P-450_{cinnamate} would undergo phytochrome-induced concn changes and that its variations would remain undetected against the bulk

phytochrome-insensitive cyt P-450. The question of multiple cyt P-450 forms and their individual evolution is central in this respect. Work concerning this point is in progress in this laboratory. The involvement of a CA4H inhibitor would provide an alternative, perhaps complementary, mechanism for the phytochrome-mediated regulation of the enzyme. Such inhibitors have been implied in the regulation of PAL [31-36] and recently evidence for an inhibitor of both enzymes in dark grown gherkin seedlings has been presented [37].

Not only red but also blue light stimulates CA4H activity in the apical bud of the pea seedling. When 8 day etiolated seedlings were given a 12 hr B irradiation, we observed the following stimulations: PAL activity $\times 3.4$, CA4H activity $\times 2$, reductase activity $\times 1.5$, cyt P-450 content $\times 1.4$, cyt *b* 5 content $\times 1.2$. So far, we have not determined the action spectrum of the blue light effect, but it was recently demonstrated that in *Neurospora crassa* [38] the reduction of *b* and *c* type cyt is mediated by a flavin. *In vitro*, the flavin sensitized photoreduction of NADP⁺ has been demonstrated [39]. The microsomal fraction contains flavins, and NADPH-cyt P-450 reductase is a flavoprotein. Further studies are needed to establish if these facts are pertinent to the mechanism of blue light induced CA4H activity stimulation.

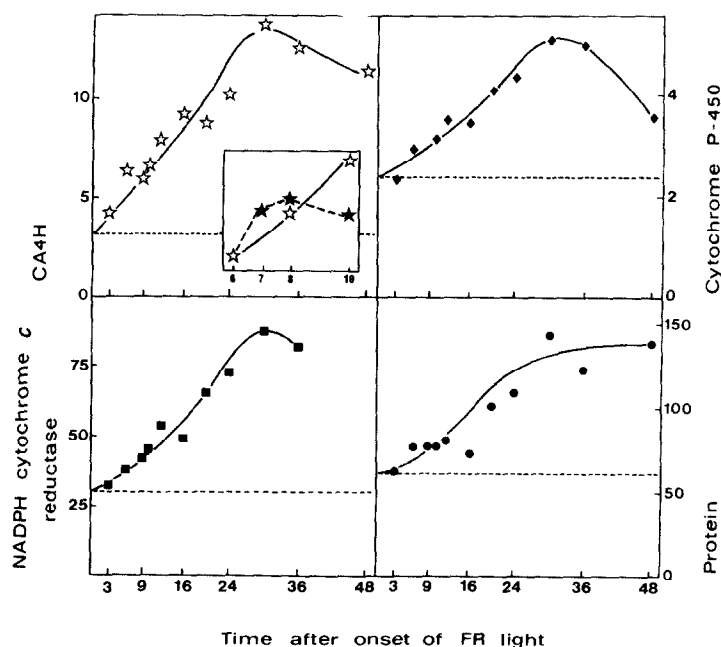


Fig. 3. Time-course of CA4H and NADPH-cyt *c* reductase activities, and cyt P-450 and protein concn, per apical bud, in response to continuous FR irradiation (in hr) of pea seedlings. CA4H (*—*) and NADPH-cyt *c* reductase (■—■) activities and concn of cyt P-450 (◆—◆) and microsomal protein (●—●) are constant in apical buds from etiolated seedlings (dotted lines), for the period considered. These values represent the average of the activities or concn of the dark extracts. The inset shows changes of CA4H activity when seedlings are returned to darkness after 6 hr FR light (★—★), compared with the activity of seedlings kept in the FR light (☆—☆).

EXPERIMENTAL

Pea seeds (*Pisum sativum* L., var. 'Téléphone à rame') were germinated and grown in moist vermiculite in darkness at 26° for 7 days. The etiolated seedlings were then irradiated with B (457 ± 20 nm), R (660 ± 10 nm) or FR (730 ± 60 nm) light. The apical buds were harvested and CA4H extracted and assayed as described previously [17]. The reaction was linear up to 20 min under these conditions. Subfractionation of a 1000 g supernatant from apical buds was achieved by isopycnic centrifugation (4 hr at 20000 rpm in a Beckman SW 27 rotor) on a continuous sucrose density gradient (30 ml) from 60–30% (wt/vol.). Sucrose soln were prepared in 0.1 M Na Pi buffer, pH 7.5, containing 1 mM EDTA. NADPH- and succinate-cyt c reductases were measured after ref. [40]. Cyt P-450 was determined by the method reported in ref. [17] using an absorption coefficient of 91 mM/cm and cyt b 5 measured from its Soret-band in the NADH reduced minus oxidized difference spectrum. UDP-galactose galactosyl-transferase was measured after [41] and catalase after [42]. For isolation of intact plastids the technique described by ref. [25], using a velocity centrifugation of a continuous and discontinuous sucrose gradient was employed. Triose phosphate isomerase activity was determined after [43] and chlorophyll concn was measured by the method of ref. [44]. Sucrose concn were determined by refractometry and protein according to [45].

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