REGULATION OF ENZYME LEVELS 1175

On the Regulation of Enzyme Levels (phenylalanine ammonia-lyase) in Different Organs of a Plant (*Sinapis alba L.*)

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The time courses of formation of the enzyme phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) mediated by far-red light (i.e. phytochrome, Pf) in cotyledons and hypocotyl of the mustard seedling (*Sinapis alba L.*) are described. While PAL activity in the cotyledons can scarcely be detected in the dark-grown seedling, the enzyme can be synthesized in the hypocotyl even in the dark. However, the degree of induction by far-red light is much greater in the cotyledons than in the hypocotyl. In the cotyledons the enzyme is not stable. The enzyme level eventually returns to nearly zero even under continuous far-red light. The time course of the level of PAL in the cotyledons (Fig. 1) can be explained by the following 3 factors (Fig. 2): 1. Induction of PAL synthesis by Pf, whereby Pf is continuously required; 2. Inactivation (degradation) of PAL by an inactivating principle; 3. Repression of PAL synthesis. The time course of the level of PAL in the hypocotyl is completely different (Fig. 1). An explanation of the hypocotyl data is presented which is based on the assumption that PAL synthesis in the dark and PAL synthesis mediated by phytochrome are independent phenomena which occur in different tissues of the hypocotyl. It appears that the occurrence of dark synthesis of a stable enzyme in the hypocotyl explains the seemingly dramatic difference between the cotyledons and the hypocotyl with respect to PAL.

Information concerning the regulation of enzyme concentrations or levels has been obtained largely from studies with microbial systems, most specifically with exponentially growing *Escherichia coli* 1, and as a rule the conclusions drawn from this information have been extrapolated to higher systems, plants and animals. On the other hand, it has been repeatedly emphasized (e.g. 2 3) that an individual plant or animal cell in a multicellular system is not comparable to the unicellular microbial system upon which most current concepts of regulation of enzyme levels have been based. In microbial systems as well as in animals or plants we must deal with the problem of how to effect changes in the metabolic machinery, i.e. specific enzymes, in response to environmental and nutritional conditions or as part of a developmental sequence. Such changes include removal of unneeded enzymes as well as the synthesis of those newly required. While in bacteria the removal process can involve dilution during phases of rapid growth, in animal or plant tissue where little or no cell division takes place the process of protein degradation is essential as a way to remove unneeded metabolic machinery, and therefore important to control enzyme levels. In animal tissues the level of enzymes can be increased by agents such as hormones, substrates, or changes in diet. However, in contrast to the general finding of stability of induced enzymes in bacteria 4, the universal finding in adult mammalian tissues has been that enzyme activity returns to a basal level along an exponential time course once the stimulus is removed 5.

In plant tissue (cotyledons of the mustard seedling) two types of enzyme have been observed in connection with phytochrome-mediated enzyme induction during photomorphogenesis. 1. Enzymes (e.g. amylase 6, glycollate oxidase 7, ascorbic acid oxidase 8) which seem to be stable during the period of experimentation; 2. Enzymes (e.g. phenylalanine

1 F. JACOB and J. MONOD, Cold Spring Harbor Symposia on Quantitative Biology 26, 193 [1961].
2 R. T. SCHMIDT, Current Topics in Cellular Regulation 1, 77 [1969].
6 M. VAN POUCKE and F. BARTH, Planta 94, 308 [1970].
7 M. VAN POUCKE, F. BARTH, and H. MOHR, Naturwissenschaften 56, 417 [1969].
ammonia-lyase\(^8\) = PAL), the activity of which returns to a low level if the light which maintains the inducing agent (active phytochrome, \(P_{Fr}\)) is turned off.

In the present paper we concentrate on the enzyme phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) from the following viewpoint: is the behaviour of this particular enzyme with respect to inducibility by phytochrome (\(P_{Fr}\)) and with respect to disappearance (degradation) the same in different organs (cotyledons and hypocotyl) of a seedling (\(Sinapis alba\) L.), or is the behaviour of the enzyme determined by the nature of the organ?

The advantages of our experimental system (mustard seedling) for the problem in question can be summarized as follows: 1. In both organs (cotyledons and hypocotyl) there is no significant increase in the amount of DNA or of cell number during the experimental period\(^9\). [Since in the taproot (= radicle) an increase in DNA and cell number must be anticipated during the experimental period, this organ was not included in the present investigation.] 2. PAL from both organs (cotyledons, hypocotyl) appears only as a single band on gel electrophoresis\(^10\). There are no indications from Schopffer's studies that the enzyme is different in the two organs\(^10\). 3. The increase in PAL activity is very probably due to an increase in the number of enzyme molecules. This problem has been repeatedly checked in the usual way, i.e. by demonstrating that drugs like, Puromycin, Cycloheximide and Actinomycin D will inhibit the increase in enzyme activity\(^11\), as well as by density-labelling with deuterium of the newly-formed enzyme\(^12\). 4. The induction of PAL by light in the mustard seedling is very probably exclusively due to \(P_{Fr}\), the active species (effector molecule) of the phytochrome system\(^13\). However, \(P_{Fr}\) can be active from the ground state (in the dark) as well as from some excited state (in continuous light)\(^14\).

Materials and Methods

Seeds of \(Sinapis alba\) L. (obtained in 1968 from Schoell, Stuttgart-Plenigen) were selected and germinated according to standard techniques developed in this laboratory\(^15\). The seedlings were grown at 25.0 ± 0.2 °C for 36 h in the dark before light treatment was started. For irradiation the standard red\(^16\), far-red\(^15\) and black-red\(^17\) sources were used at an irradiance of 67.5 \(\mu\)W·cm\(^{-2}\) ± 10% (red), 350 \(\mu\)W·cm\(^{-2}\) ± 10% (far-red) and 700 \(\mu\)W·cm\(^{-2}\) ± 10% (black-red, 756 nm) at 25.0 ± 0.5 °C. The standard methods of extraction and assay of PAL described elsewhere\(^14\) were used. In addition a modification of the method developed by Schopffer\(^14\) was applied. While this procedure gives some increase in sensitivity over the usual assay, it did not alter the results. For the sake of simplicity the standard method was retained. The values presented are of 4 to 32 independent experiments. Standard errors were in the range of 2.5 to 10% unless indicated otherwise. The use of the biological unit (cotyledon, hypocotyl) as a system of reference was justified previously\(^6\). Due to an error of calculation which was only detected in the course of the present investigation, all values of PAL which we have published since 1966\(^18\) (except\(^14\)) must be multiplied by 3 in order to obtain the actual values of activity. The use of continuous standard far-red light was repeatedly justified previously (e.g.\(^14\),\(^19\)). In the cotyledons of the mustard seedling this light maintains a low but virtually stationary concentration of the active phytochrome (\(P_{Fr}\)) during the whole experimental period\(^20\), whereas in the hypocotyl the phytochrome concentration decreases slowly but steadily during the experimental period\(^21\). The photoequilibrium that is characteristic for the standard far-red light is established in both organs in a matter of a minute after the onset of far-red light.

Results and Interpretation

**PAL levels in the cotyledons** (Fig. 1)

In the dark-grown mustard cotyledons PAL activity can scarcely be detected by our assay. However, the enzyme can rapidly be induced by continuous far-red light (i.e. by \(P_{Fr}\), cf. Methods). Experiments with inhibitors of RNA and protein

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13. K. M. Hartmann, Naturwissenschaften 54, 544 [1967].
15. H. Mohr, Z. Pflanzenphysiolog. 54, 63 [1966].
synthesis indicate that the far-red-mediated increase of enzyme activity in the mustard seedling is due to an increase in the number of enzyme molecules (Table 1). This result confirms the conclusion drawn by SCHOPPER and HOCK, who have recently applied the in vivo density labelling technique with deuterium oxide to PAL in the mustard cotyledons.

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>per cent inhibition of PAL-increase</th>
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<tbody>
<tr>
<td>Actinomycin D [10 µg·ml⁻¹]</td>
<td>0</td>
</tr>
<tr>
<td>Puromycin [100 µg·ml⁻¹]</td>
<td>67</td>
</tr>
<tr>
<td>Cycloheximide [5 µg·ml⁻¹]</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 1. The effect of inhibitors of RNA- and protein synthesis on the far-red-mediated increase of PAL in the total mustard seedling over a period of 12 h after the onset of continuous far-red light. The application of the drugs was carried out according to the method of LANGE et al. during a 1 h submerged incubation in the dark of 35-h-old seedlings. Control seedlings were incubated in distilled water. After treatment, the seedlings were returned to standard dishes. Onset of continuous far-red light: 36 h after sowing. Enzyme assay: 60 h after sowing.

Our problem is to explain the basic kinetics, i.e., the time course of PAL levels in the mustard cotyledons under the influence of continuous far-red light. The experimental basis of any explanation of the basic kinetics is found in the far-red → dark kinetics elaborated up to the peak of the basic kinetics (Fig. 1). The theoretical basis of these experiments (as far as phytochrome is concerned) is briefly the following: When the far-red light is turned off, the physiological effectiveness of the established \( P_f \) concentration drops instantaneously to a low level. The reason for this change is that the physiological effectiveness of a steady state \( P_f \) concentration (maintained by standard far-red light) is a function of the quantum flux density over a wide range. HARTMANN has explained the irradiance dependency by the hypothesis that \( P_f \) can act from some excited state, \( P_f^* \), which is more active physiologically than \( P_f \) in the ground state. When the far-red light is turned off, the \( P_f^* \) species disappears almost instantaneously, while the physiologically much less effective \( P_f \) molecules in the ground state only disappear with a half-life of about 45 minutes. Table 2 shows that those \( P_f \) molecules (ground state) which remain in the cotyledons after the standard far-red light has been turned off, promote the increase of PAL only slightly during the following 3 h as compared to a continuous irradiation with standard far-red light. As a basis of reference we must choose the value which we determine after a posttreatment with black-red (lower line). The reason is that a posttreatment with 5 min of black-red (756 nm) reduces the \( P_f \) concentration to a very low level, an thus leads to a decrease in the PAL level, while a posttreatment with 5 min of red ([\( P_f \)]/\([P_{total}]\) ≈ 0.8) actually leads to a transient increase in the PAL level. These facts are in ac-

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22 H. LANGE, I. BIENGER, and H. MOHR, Planta 76, 359 [1967].
The basic kinetics of the PAL levels in the cotyledons of the mustard seedling (dashed line) can be explained by an interaction of the following 3 factors: enzyme induction, enzyme degradation (inactivation), repression of PAL synthesis. The beginning and (in the case of the first factor) the end of the period of involvement of the factors are indicated by the vertical bars.

Table 2. The effect of a posttreatment with 5 min of red light or 5 min of black-red (756 nm) light on the level of PAL 3 h after the termination of a 12 h treatment with continuous standard far-red light. The posttreatment with red or black-red light was carried out immediately after the end of the far-red treatment. Note that the responsiveness of the seed sample used in these experiments differed somewhat from the responsiveness of the sample used previously (cf. Fig. 1). However, in essence the results are not affected by these temporal changes of responsiveness of the seed population.

<table>
<thead>
<tr>
<th>treatment after sowing (d = dark; fr = far-red)</th>
<th>PAL activity pmoles trans-cinnamic acid min·pair of cotyledons</th>
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<tbody>
<tr>
<td>36 h d + 12 h fr</td>
<td>66.2 ± 2.2</td>
</tr>
<tr>
<td>36 h d + 12 h fr + 3 h fr</td>
<td>104.1 ± 2.1</td>
</tr>
<tr>
<td>36 h d + 12 h fr + 3 h d</td>
<td>66.0 ± 4.0</td>
</tr>
<tr>
<td>36 h d + 12 h fr + 5 min red + 3 h d</td>
<td>75.3 ± 2.9</td>
</tr>
<tr>
<td>36 h d + 12 h fr + 5 min 756 nm + 3 h d</td>
<td>58.2 ± 3.4</td>
</tr>
</tbody>
</table>

In the hypocotyl (as well as in the taproot) PAL appears in the dark. Furthermore, the basic kinetics of PAL, i.e., the time course of PAL levels under continuous standard far-red light, are totally different as compared to the basic kinetics in the cotyledons. Our preferred explanation of the hypocotyl data is based on the assumption that PAL synthesis in the dark and PAL synthesis mediated by phytochrome are independent phenomena. If we subtract the dark kinetics from the far-red kinetics we find a time course of the rest enzyme which is similar to the far-red kinetics in the cotyledons and possibly explainable in the same way (cf. Fig. 2). Since the dark enzyme and the \( P_{fr}\)-dependent enzyme do not differ in gel electrophoresis and in every other aspect tested so far, the conclusion is justified that the two enzymes are identical. The situation in the hypocotyl can then be understood as follows: There are tissues in the hypocotyl (e.g., in the differentiating xylem) which produce the enzyme in the dark. This enzyme is stable, at least during the

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period of experimentation. On the other hand, there are tissues in the hypocotyl (e.g. the anthocyanin synthesizing subepidermal layer) which produce the enzyme only under the control of $P_{fr}$, as is the case in the cotyledons. These two responses are independent of one another. If this interpretation is correct, the only difference between the cotyledons and the hypocotyl with respect to PAL would be that in the one organ there is considerable dark synthesis of a stable enzyme while in the other organ dark synthesis of PAL is scarcely detectable.

While it is possible to interpret the PAL data of Fig. 1 with more complicated models 28, the basic conclusion can hardly be circumvented. Although there is an obvious and seemingly complicated difference in the behaviour of this particular enzyme in the two organs, it is highly probably that this difference is mainly (or even exclusively) due to the existence of a considerable dark synthesis of the enzyme in the hypocotyl. The factors controlling dark synthesis in our system are unknown 29.

**General Discussion**

While the behaviour of PAL in the organs of the radish seedling 30 is possibly similar to the behaviour of PAL in the mustard seedling, a different situation was reported for PAL in dark-grown buckwheat seedlings 31. In this plant PAL activity increased in continuous darkness in all organs of the seedling and reached a maximum on the 4th day after sowing which was followed by a rapid decrease. Furthermore, the light-mediated induction of PAL in the buckwheat hypocotyl 32 follows kinetics which are more similar to those in mustard cotyledons than in the hypocotyl. ENGELLSMA 33 has recently compared the control of PAL activity in red cabbage hypocotyl with that in gherkin seedlings. He found that in red cabbage hypocotyls the photoinduced increase in the activity of PAL was not followed by a decline as was previously 34 described for gherkin seedlings. ENGELLSMA concluded that a PAL-inactivating system such as is assumed to exist in gherkin hypocotyls does not operate in red cabbage hypocotyls. From these and from the present data we have to conclude that the pattern of regulation of PAL is different in different plant species.

Our previous conclusion, namely, that in connection with enzyme induction $P_{fr}$ more or less directly interacts with the genetic material 35, 36, has been supported by recent data. Two examples may be mentioned: The rapidity of the response (a change in $P_{fr}$ will lead almost instantaneously to a change in PAL synthesis if $P_{fr}$ is the only limiting factor of PAL synthesis 41) has been confirmed 37; and HADWIGER and SCHWOCHAU 38, 39 have shown that most of the inducers of PAL in pea pod tissue have the potential to change the conformation of DNA (DNA intercalating compounds). These authors have suggested that the control of PAL synthesis occurs at the gene transcription level and involves a conformational change of the double-stranded DNA. The induction of PAL by ultraviolet light has also been interpreted in terms of conformational changes in segments of the DNA 40.

Cycloheximide not only inhibits the initial synthesis of PAL but also inhibits inactivation of the enzyme if added at the beginning of the dark period 41, 42, 43. This finding has been interpreted to mean that de novo synthesis of some protein(s) is required for inactivation. The question is whether the rate of inactivation at a given time (e.g. 12 h after the onset of far-red light) remains the same after the light is turned off 44. While recent results

28 M. ZUCKER, Plant Physiol. 47, 442 [1971].
30 E. BELLINI and M. VAN POUCKE, Planta 93, 60 [1970].
31 N. AMRHEN and M. H. ZENK, Z. Pflanzenphysiol. 63, 384 [1970].
32 N. AMRHEN and M. H. ZENK, Z. Pflanzenphysiol. 64, 145 [1971].
34 G. ENGELLSMA, Planta 75, 207 [1967].
41 G. ENGELLSMA, Naturwissenschaften 54, 319 [1967].
42 M. ZUCKER, Plant Physiol. 43, 365 [1968].
46 M. ZUCKER, Plant Physiol. 47, 442 [1971].
obtained by ZUCKER with green leaf disks from the cocklebur plant, floating on sucrose solution [43], suggest that the rate of inactivation increases in darkness, we feel that in the case of the mustard seedling the data (e. g. Fig. 1) indicate that the P4-mediated increase of PAL is due to an increase of enzyme synthesis rather than to a decrease of the rate of PAL inactivation. It is difficult to settle this question with more direct experiments since chase experiments with pulse-labelled PAL have led to results which are difficult to interpret [46].

From this paper it is evident that the regulation of enzyme levels in organized plant tissues is complex, and that multiple mechanisms exist for controlling the level of a particular enzyme in a particular organ. Obviously there is no single or simple mechanism which controls the levels of an enzyme in all instances. As in the case of mammalian tissues we are led to the conclusion that control of the level of a particular enzyme may be exerted at any point at which control can be potentially exerted, and this will depend upon the enzyme and the tissue involved.

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Konzentration einiger Lipide in den Chloroplasten von Zea mays und Antirrhinum majus

Concentration of Some Lipids in the Chloroplasts of Zea mays and Antirrhinum majus

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Chloroplasten Lipide

Stroma-containing chloroplasts from Zea mays and Antirrhinum majus were isolated in aqueous medium. The average dry weight of chloroplasts from Zea mays is 27·10^{-12} g, that of Antirrhinum majus 30·10^{-12} g. Water freed chloroplasts consist up to 49 or 45 percent respectively of lamellar system. The lipid content of the lamellar system of Zea mays is 49 percent, that of Antirrhinum majus 45 percent. A chloroplast of Zea mays contains on the average 920·10^{15} chlorophyll molecules, 220·10^{15} carotenoid molecules, 2000·10^{15} molecules of galactolipids, 190·10^{15} molecules of sulpholipid, 260·10^{15} phosphatide molecules and 64·10^{15} molecules of lipophilic quinones. In addition to phosphatidylglycerol also phosphatidylinositol and phosphatidylcholine were found. It is very probable that besides vitamin K_3 the homologous compound lacking one methylgroup is present in the chloroplasts. In contrast to the literature only 62 percent of the total leaf galactolipids are found in the chloroplasts.

Bei den ersten Versuchen, Chloroplasten zu isolieren, stand der Gesichtspunkt im Vordergrund, Präparate zu erhalten, die nicht durch andere Zellbestandteile verunreinigt waren. Daher wurden Präparate, deren Chlorophyllgehalt möglichst hoch war und sich bei fortgesetzter Fraktionierung nicht mehr steigern ließ, als rein angesehen. Erst als TREBST, TSUJIMOTO und ARNON zeigten, daß das Stroma bei der Isolierung der Chloroplasten leicht austritt und beim fraktionierten Zentrifugieren im Überstand bleibt, wurde klar, daß ein hoher Chlorophyllgehalt nicht ohne weiteres als Kriterium für die Reinheit eines Chloroplastenpräparates angesehen werden kann. Heute weiß man, daß bei den ersten Isolierungsversuchen vorwiegend stroma freie Chloroplasten erhalten wurden. Auch in neueren Veröffentlichungen ist es häufig nicht ersichtlich, ob sich die Angaben auf Chloroplasten oder auf ihr Lamellar- system beziehen. Um Stromaverluste zu vermeiden, verwendeten BEHRENS und THALACKER, HEBER.