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PROPERTIES OF L-PHENYLALANINE AMMONIA-LYASE AND TURNOVER RATE IN ETIOLATED AND FAR-RED ILLUMINATED SEEDLINGS OF RADISH

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Summary

1. L-Phenylalanine ammonia-lyase (EC 4.3.1.5) was purified from radish cotyledons. The adsorption of the enzyme on L-phenylalanine-Sepharose 4B was nonspecific; the present data disprove the conclusions reached previously (Blondel, J.D., Huault, C., Faye, L., Rollin, P. and Cohen, P. (1973) FEBS Lett. 36, 239—244).

2. The apparent molecular weight of the enzyme fluctuated according to pH and ionic strength. A mean value of 290 000 was determined for the 'native' form. The activation energy was 54.4 kJ/mol. L- and D-phenylalanine protected the lyase from sodium borohydride denaturation.

3. Two Michaelis constants, $K_m^H = 1.5 \cdot 10^{-5}$ M and $K_m^L = 9.5 \cdot 10^{-5}$ M, were determined. The Hill coefficient value (h) was 0.48. This coefficient was 0.6 for cinnamate inhibition. These results suggest that phenylalanine ammonia-lyase in radish cotyledons is regulated by a negative cooperativity mechanism.

4. The phytochrome-mediated increase in phenylalanine ammonia-lyase activity was investigated by density labelling with ^2H_2O followed by isopycnic centrifugation in KBr gradients. An apparent half-life of 3.5 h was found for both the enzyme extracted from etiolated or far-red irradiated cotyledons.

5. Provided that phytochrome does not control the availability of labelled amino acids for protein synthesis, the present results are consistent with a far-red light-induced increase in the rate of synthesis of the lyase in radish cotyledons.

Introduction

L-Phenylalanine ammonia-lyase (EC 4.3.1.5) is an enzyme catalysing the deamination of L-phenylalanine to form *trans*-cinnamic acid [1]. The enzyme is widely distributed amongst plants, from fungi to higher plants [2,3], and has been purified to homogeneity from potato tubers, maize shoots [4], wheat seedlings [5] and parsley cell cultures [6]. Most authors agree that the enzyme consists of four subunits. The activity of phenylalanine ammonia-lyase fluctuates in response to a variety of stimuli such as wounding [7,8], viral infection [9,10], growth inducers [11], ethylene [12] and light. The latter stimulus has been studied most and it has been established that any specific increase in phenylalanine ammonia-lyase activity is under the control of phytochrome [13,15]. The mechanism by which this control is exercised is still in debate [16,19]. The main reason for disagreement between authors arises from the methods used for investigation, i.e. the density labelling technique.

As enzyme purification is not a prerequisite for heavy-isotope labelling [20], such labelling was thought to be the appropriate method for the study of the regulation of phenylalanine ammonia-lyase synthesis. Unfortunately, although density labelling is a powerful tool in DNA replication studies, its resolution is lower for smaller molecules such as proteins [21]. We reported recently [22] the advantages of using KBr instead of CsCl in isopycnic centrifugation of proteins, and this method was therefore used in the present study.

The work reported here was undertaken to throw light on the question of whether phytochrome stimulates phenylalanine ammonia-lyase synthesis in radish cotyledons. Density labelling results as well as reexamination of the enzyme purification in this material seem to positively answer that question. We also report on the enzyme's properties, the molecular weight, activation energy, borohydride denaturation, and kinetic behaviour.

Materials and Methods

Plant growth and labelling conditions. Seeds of radish (*Raphanus sativus*, var. Longue Rave saumonée) were sown on moist filter paper and germinated in the dark for 36 h at 25 °C. Unless otherwise stated they were then transferred to standard far-red light as described previously [24]. This light treatment yields a maximal increase in extractable phenylalanine ammonia-lyase in this material [15]. When appropriate, seedlings were transferred to dry filter paper for removal of excess water and then to filter paper imbided with ^2H_2O.

Preparation of enzyme extracts for isopycnic centrifugation. 200 cotyledons were harvested and homogenized in an ice-cold mortar in 0.1 M acetate buffer, pH 6. Crude extracts were centrifuged for 20 min at $10\,000 \times g$ and the resulting supernatant was desalted through Sephadex G-25. Partial purification was then carried out on Sepharose 4B-L-phenylalanine; the enzyme was eluted from the column with 0.1 M borate buffer, pH 7.9.

Isopycnic centrifugation. KBr gradients were prepared as described previously [22]. Centrifugation was carried out in a Beckman L5 75 ultracentrifuge using a type 75 Ti fixed angle rotor operated at $182\,000 \times g_{av}$, (r_{av} = 5.9 cm) and 6 °C for 20 h. Every tenth fraction (approx. 90 µl) from the gradients was

used for refractive index determination using an Abbe 60 refractometer; these values were then converted into density units by using the equation of Vinograd and Hearst [25]. Other fractions were assayed for phenylalanine ammonia-lyase activity.

Phenylalanine ammonia-lyase assay. The spectrophotometric assay was based on Zucker's procedure [7]. 0.5 ml enzyme extract was added to a mixture of 2 ml 0.05 M borate buffer (pH 8.8) and 0.5 ml 0.1 M L-phenylalanine made up in the same buffer. The increase in absorbance at 290 nm after incubation at 80 °C was recorded against a blank which lacked substrate. Incubation time was 24 or 36 h for fractions from KBr gradients and usually 1 h in other cases. The radiochemical assay was as described by Attridge and Smith [26]. For routine determination of active fraction after a purification step, we used the following method. To an 0.2 ml aliquot, 2 ml 0.05 M borate buffer (pH 8.8) containing 20 mM L-phenylalanine were added. After incubation at 30 °C for 15 min, the enzyme activity was detected by adding 0.5 ml 0.1% $KMnO_4$. The reduction of $KMnO_4$ by cinnamic acid resulted in a yellow coloration in active fractions.

Miscellaneous. Sepharose 4B was covalently coupled to L-phenylalanine according to Axen et al. [27]. Electrophoresis was carried out in 8% acrylamide

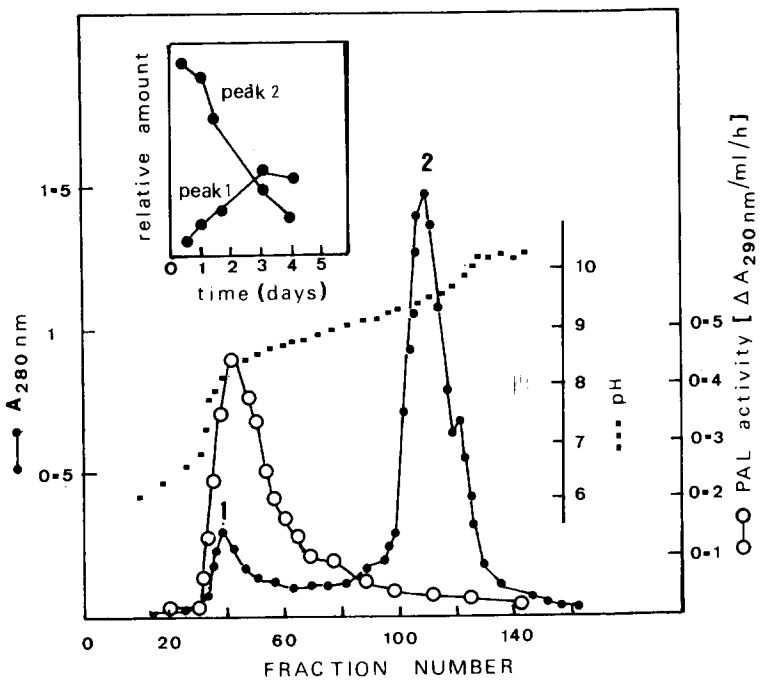

Fig. 1. Sepharose 4B-L-phenylalanine chromatography of phenylalanine ammonia-lyase of radish cotyledons. The enzyme extract (approx. 300 ml) was applied to a column (9 × 2.5 cm) of L-phenylalanine-Sepharose 4B pre-equilibrated with 0.1 M acetate buffer, pH 6. The sample was allowed to pass through the gel and the column was rinsed with 0.1 M acetate buffer (pH 6) until the A_{280} nm of the effluent returned to zero. Elution was achieved with a pH gradient (6—10). PAL, the activity of phenylalanine ammonia-lyase (○———○). Proteins were monitored at 280 nm (●———●). Fractions 30—60 were pooled for further purification. The insert represents the relative changes (arbitrary units) of the two protein peaks obtained with extracts of cotyledons of far-red light grown seedlings within the first 6 days of germination; t_0 is 36 h after sowing.

gels using a acrylamide/bisacrylamide ratio of 30 : 0.8 w/w) [28]. Thyroglobulin, β-galactosidase, myoglobin and cytochrome *c* were purchased from Sigma. The L-[U-^{3}H]phenylalanine was obtained from the Radiochemical Centre, Amersham, U.K., or C.E.A., Saclay, France.

Purification of phenylalanine ammonia-lyase. All steps were carried out at 4°C. 10—30 g cotyledons were homogenized in a Waring blendor set at high speed for 4 × 15 s in 0.1 M acetate buffer, pH 6. After filtration through four layers of cheese-cloth, the homogenate was centrifuged for 20 min at 15 000 × *g*. The supernatant was then desalted through a Sephadex G-25 column (5 × 110 cm) equilibrated with 0.1 M acetate buffer, pH 6. The Sephadex G-25 extract was then subjected to chromatography on Sepharose 4B coupled to L-phenylalanine as first described by Blondel et al. [23]. The apparently homogeneous fraction obtained when the elution was carried out using a pH shift of 4 units was resolved into several components when elution was achieved by a pH gradient. Two main peaks are observed (Fig. 1). Only the first minor peak exhibited phenylalanine ammonia-lyase activity. The second peak possibly consists of the major reserve protein of radish seeds, since its contribution decreased gradually during the first days of germination (Fig. 1). It is very likely that this fraction was though to be the phenylalanine ammonia-lyase in the investigation of Blondel and coworkers [23]. Though we probably failed in doing affinity chromatography, this step was quite efficient and yielded a 30-fold purification. Active fractions were pooled and concentrated

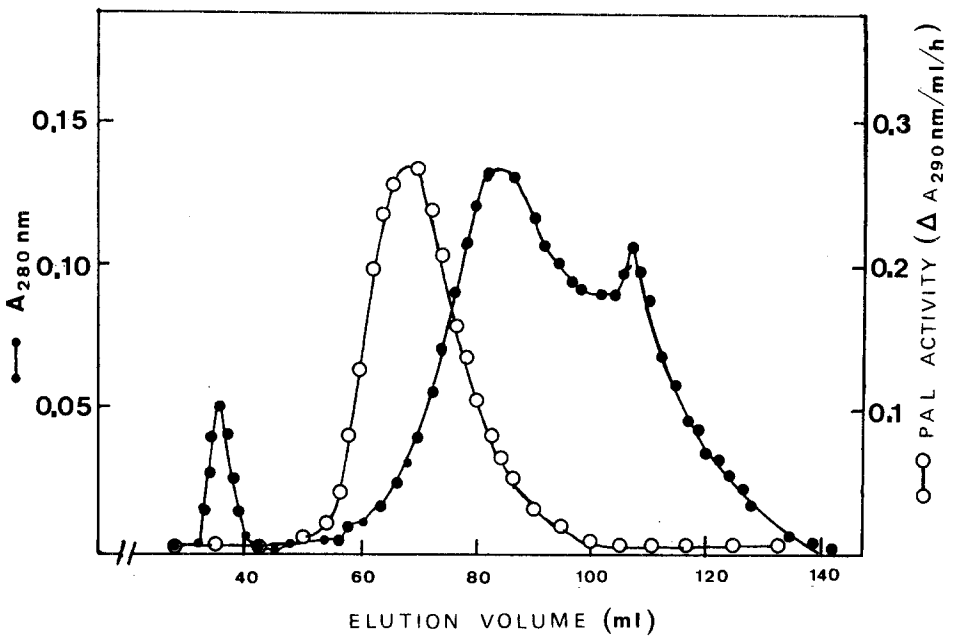

Fig. 2. Sepharose 6B chromatography of phenylalanine ammonia-lyase. Active fractions from Sepharose 4B-L-phenylalanine chromatography (see Fig. 1) were concentrated and introduced onto Sepharose 6B column (70 × 1.5 cm) equilibrated with 0.1 M acetate buffer, pH 6. Size of sample was 0.5 ml; flow rate 0.5 ml/min; protein load; approx. 10 mg. Fractions were assayed for proteins (●———●) and phenylalanine ammonia-lyase activity (PAL) (○———○). Fractions 60—75 were pooled for further analysis.

by ultrafiltration (Amicon XM 100). The enzyme was further purified by molecular sieving through a Sepharose 6B column (Fig. 2). Obviously, phenylalanine ammonia-lyase represents a minor component of the first peak eluted from the Sepharose 4B-L-phenylalanine. Neither electrophoresis nor DEAE-cellulose chromatography revealed the existence of more than one form of active phenylalanine ammonia-lyase. In a typical experiment the specific activity rose from 3.3 mkat/kg proteins to 93 mkat/kg for the fraction eluted from the Sepharose 4B-L-phenylalanine and to 790 mkat/kg after the Sepharose 6B chromatography. The enzyme was purified 240-fold with a 27% yield.

Results

General properties and kinetic parameters

Fig. 3 represent the elution of phenylalanine ammonia-lyase on a Sepharose

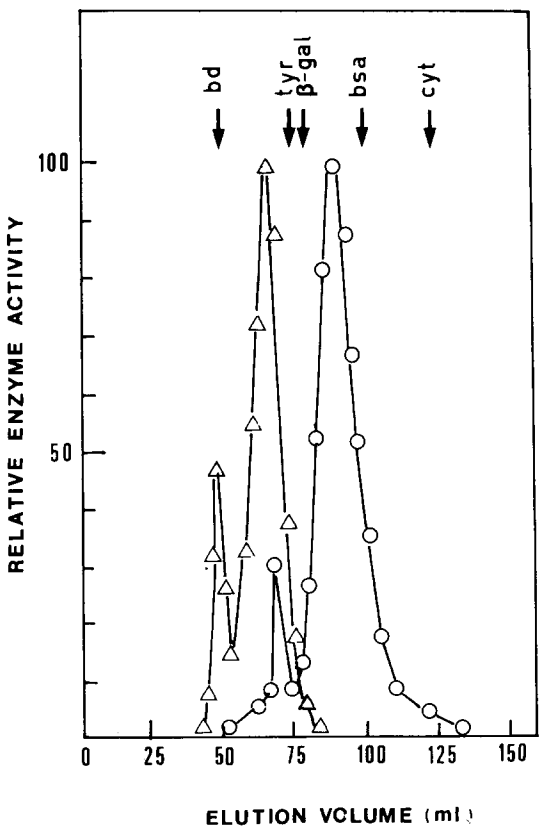

Fig. 3. Molecular weight determination of phenylalanine ammonia-lyase of radish cotyledons. 0.5 ml (approx. 10 mg protein) enzyme extract (concentrated pooled fractions as indicated in Fig. 1) were applied to a Sepharose 6B column (70 × 1.5 cm) equilibrated either with 0.05 M borate buffer, pH 8.8 (△———△), or with 0.1 M acetate buffer, pH 6 (○———○). Flow rate was 0.5 ml/min. Arrows indicate the elution volume of the markers: cytochrome *c* (cyt *c*, 12 000), bovine serum albumin (bsa, 55 000), β-galactosidase (β-gal, 540 000), thyroglobulin (tyr, 650 000) and blue dextran (bd).

6B column calibrated with five protein markers. When the enzyme was filtered in 0.05 M borate buffer (pH 8.8), the apparent molecular weight was found to be above 1 000 000. In 0.1 M acetate buffer (pH 6), a value of 290 000 ± 5% was recorded. Increasing the ionic strength up to 0.6 M KCl led to an apparent molecular weight of 140 000. These last data should be interpreted with caution, since Lin and Castell [29] demonstrated that in buffer of high ionic strength the physical properties of Sepharose are modified. Experiments conducted on Sephadex G-200 using 0.05 M borate buffer (pH 8.8) containing or lacking 0.6 M KCl led to similar results. Therefore it is likely that ionic strength is the main factor controlling the molecular aggregation of the enzyme; the possible role of pH was not investigated further. A molecular weight of 290 000 is in good agreement with other reports on phenylalanine ammonia-lyase from various sources [6,30—32]. Unlike the enzyme prepared from a microbial source [33,34], the phenylalanine ammonia-lyase of radish cotyledons did not deaminate L-tyrosine. The activation energy determined by using the equation of Arrhenius [35] was 54.4 kJ/mol. The enzyme was protected from $NaBH_4$ denaturation by L- and D-phenylalanine. This is consistent with the presence of an essential dehydroalanine at the active site [36].

Determination of initial velocities versus substrate concentration showed that phenylalanine ammonia-lyase did not follow a typical Michaelis-Menten kinetics. Using the terminology of Datta and Gest [37] the K_m^H and K_m^L values were $1.5 \cdot 10^{-5}$ M and $9.5 \cdot 10^{-5}$ M, respectively. A Hill coefficient of 0.48 was determined when data were plotted according to Atkinson et al. [38]. This coefficient was 0.6 when cinnamic acid was used as an inhibitor.

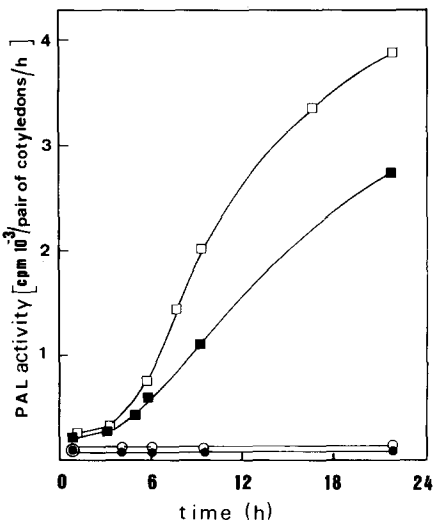

Fig. 4. Time course of phenylalanine ammonia-lyase activity in radish cotyledons; effect of ^2H_2O. Seeds of radish were germinated for 36 h in the dark on H_2O. They were then transferred either to far-red light and on H_2O (□), far-red light and ^2H_2O (■), in the dark and on H_2O (○) or in the dark and a ^2H_2O (●). The enzyme was assayed by the radiochemical method. t_0 is 36 h after sowing.

Effect of ^2H_2O on the developmental changes in phenylalanine ammonia-lyase

Fig. 4 shows the comparative evolution of the phenylalanine ammonia-lyase activity in cotyledons of seedlings grown either on H_2O of ^2H_2O (approx. 100%). The level of extractable enzyme was lower in the case of exposure to ^2H_2O; however, the developmental pattern was not affected. Therefore, undiluted ^2H_2O was used in this work.

Turnover rate of phenylalanine ammonia-lyase activity in cotyledons of etiolated seedlings

Fig. 4 shows that phenylalanine ammonia-lyase in cotyledons of etiolated radish seedlings remains at an apparent steady-state level, in contrast with the large increase induced by far-red light. Despite the relatively low level of phenylalanine ammonia-lyase activity in darkness, it was still feasible to assay accurately the enzyme after isopycnic centrifugation. This was made possible by the partial purification of the extracts, which prevented overloading of the gradients.

The kinetics of ^2H incorporation into phenylalanine ammonia-lyase are presented in Fig. 5. It is clear that a synthesis of the enzyme occurs in darkness. A maximal band-broadening was observed after 3 h of incubation with ^2H_2O.

Effect of far-red light on the turnover of phenylalanine ammonia-lyase

In order to investigate whether phenylalanine ammonia-lyase is synthesized as an inactive enzyme which is activated by light via phytochrome [16], the following experiment was designed. Seedlings of radish were germinated for 36 h in the dark on H_2O and transferred onto ^2H_2O for 24 h. They were then

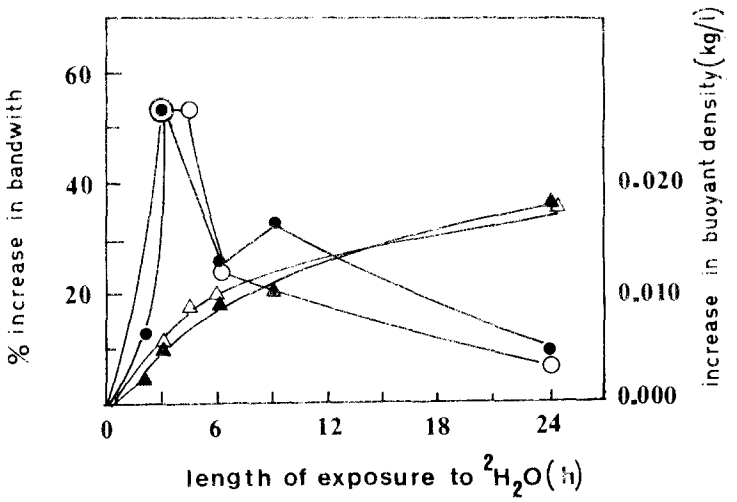

Fig. 5. Kinetics of ^2H labelling of phenylalanine ammonia-lyase in radish cotyledons. Seeds were germinated for 36 h in the dark on H_2O. Seedlings were then either left for 6 h on H_2O under far-red light and transferred onto ^2H_2O in continuous far-red light (open symbols) or transferred onto ^2H_2O in darkness (closed symbols). t_0 is 36 h after sowing for the dark treatment and 36 + 6 h for the pre-irradiated seedlings. Percentage of increase in bandwidth (circles) and increases in buoyant density (triangles) were recorded.

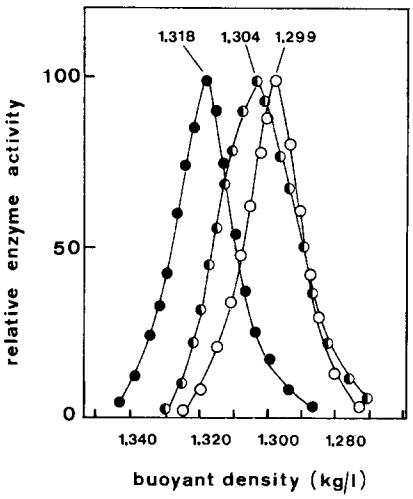

Fig. 6. Isopycnic centrifugation of phenylalanine ammonia-lyase. Seeds of radish were germinated for 36 h on H_2O in darkness. Seedlings were then given the following treatments: on H_2O for 24 h in darkness (open circles); on ^2H_2O for 24 h in darkness (closed circles); on ^2H_2O for 24 h in darkness and on H_2O for 6 h under far-red light (half-closed circles).

replaced on H_2O and simultaneously given 6 h far-red irradiation. If the increase in phenylalanine ammonia-lyase activity observed in the irradiated cotyledons is mainly due to the activation of an inactive protein synthesized in darkness, this should result in a buoyant density of the enzyme close to that obtained after a 24 h labelling period in darkness. Fig. 6 shows that this was not the case. The buoyant density shift was 0.005 kg/l compared to 0.018 kg/l for the control.

We compared the labelling of phenylalanine ammonia-lyase extracted from cotyledons of etiolated seedlings with that of the pre-irradiated seedlings [18]. Results are presented in Fig. 5. No significant difference was observed between the results of the two treatments, neither in buoyant density nor in bandwidth change. Furthermore, when seedlings were put in darkness for 3 h on ^2H_2O after 6 h of far-red light irradiation on H_2O, the distribution of the enzyme after isopycnic centrifugation was similar to that obtained when seedlings were exposed simultaneously to far-red light and ^2H_2O after the pre-irradiation. The apparent half-life of phenylalanine ammonia-lyase was estimated by calculating the percentage of remaining unlabelled enzyme after various periods of exposure to ^2H_2O. A value of 3.5 h was found for the enzyme extracted from both etiolated and far-red illuminated cotyledons.

Discussion

In a previous report of Blondel et al. [23] it was concluded that phenylalanine ammonia-lyase from radish cotyledons was purified to homogeneity in a one-step procedure, chromatography on Sepharose 4B-L-phenylalanine. This statement relied on the assumption that affinity chromatography was achieved. The present reinvestigation demonstrates that phenylalanine ammonia-lyase

was not selectively bound to such an adsorbant. Two reasons can account for this. Firstly, the cyanogen bromide treatment creates charged groups on the matrix. Secondly, the presence of the aromatic ring of phenylalanine may establish hydrophobic interactions with proteins [40]; and indeed, the Sepharose 4B-L-phenylalanine used in this work is closely related to the commercially available support for hydrophobic chromatography, i.e. phenyl-Sepharose (Pharmacia Fine Chemicals). Tanaka and Uritani [41] using a similar procedure for isolating phenylalanine ammonia-lyase from sweet potatoe, reported that the elution of the enzyme could be achieved by a gradient of 1—10 mM phenylalanine. However, the pH of eluting buffer was simultaneously shifted 3 units. Our results indicate that pH change alone was able to release the enzyme from the matrix. Neither 10 mM L-phenylalanine nor 1 M NaCl were able to elute phenylalanine ammonia-lyase. The properties of the lyase of radish cotyledons are similar to those of enzymes extracted from different sources [2,3,6,30]. Occurrence of high molecular weigth aggregates (above 1 000 000) was also demonstrated for phenylalanine ammonia-lyase of mustard seedlings [32] and of parsley cell cultures [6]. Zimmermann and Hahlbrock [6] suggested that this could be attributed to the existence of different forms of the enzyme. Our data show that the two (or more) molecular forms are interconvertible by means of ionic strength changes. The main feature of phenylalanine ammonia-lyase is revealed by initial velocity studies, that is, the non-Michaelian behaviour. Observed characteristics, i.e., two K_m values and a Hill coefficient close to 0.5 meet the criteria for negative cooperativity [42, 43]. Negative cooperativity, which seems to be the rule for phenylalanine ammonia-lyase [6,30,34], was not observed for the enzyme extracted from mustard cotyledons [44]. Since the previous procedure for determination of phenylalanine ammonia-lyase from radish cotyledons [23] was misleading, the hypotehsis of the so-called inactive form of the enzyme was no longer valid. We reinvestigated the mechanism of the regulation of phenylalanine ammonia-lyase by phytochrome by using the density labelling technique. The work described here differs from previous investigations in two ways. (i) Partially purified enzyme extracts were used instead of crude extracts, thus allowing a reduction in the protein loading of gradients and limiting the possible binding of phenolic compounds to proteins [44,45]. This might be of importance since the amount of phenolic products is phytochrome-dependant [46]. (ii) Analysis of labelled proteins was carried out in KBr instead of CsCl gradients [22]; it is worth noting that changes in band-width reported here are greater than those determined in previous studies [17,18].

The concentration of ^2H_2O to be used for labelling proteins has been discussed extensively [17,18,47]. It is clear that a high concentration of ^2H_2O in the growth medium reduces many biological activities [48,49]. On the other hand, lowering the percentage of ^2H_2O results in a reduction of the extent to which proteins are labelled [50]. Recently, Lamb et al. [47] found that the inhibition of protein synthesis caused by isotopic stress was the limiting factor for ^2H labelling. We used high ^2H_2O concentration because preliminary experiments did not reveal any dramatic effect of ^2H_2O on the evolution of lyase activity within the first 10—12 h. As pointed out by several authors [17—19], the level of deuteration of the amino acid pool is an essential factor

conditioning the validity of density labelling experiments. Unfortunately, the rate of labelling of the amino acid pool with ^2H_2O during the development of radish cotyledons is not known. An attempt to measure this rate in mustard cotyledons was made by Johnson and Smith [19]. They found that it could be controlled by phytochrome; however they used an indirect procedure which had relatively poor sensitivity. Moreover, one may argue that extractable amino acids are not necessarily available to protein synthesis. At present we cannot exclude the possibility that light affects the rate of labelling of the amino acid pool in our material.

The present data (Fig. 6) seem to rule out the hypothesis of an inactive precursor of phenylalanine ammonia-lyase in radish cotyledons. The apparent half-life of assayable lyase was estimated 3.5 h, which is consistent with the results of Tong and Schopfer [18]. They found a shorter half-life of the enzyme under far-red irradiation, whereas we did not observe any significant difference between dark and light treatment. We suggest two possible explanations for our data. The first is that the enzyme is synthesized in all cells of the cotyledons in darkness, and that far-red light, acting through phytochrome, would increase the rate of both synthesis and degradation. Alternatively, phytochrome could promote the synthesis of the lyase in those cells which do not produce it in darkness, the turnover rate being the same in all cells. Both explanations imply that far-red light does not control the availability of labelled amino acids for protein synthesis. This has not been investigated in the present study.

Acknowledgements

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References

1 Koukol, J. and Conn. E.E. (1961) J. Biol. Chem. 236, 2962—2698
2 Camm, E.L. and Towers, G.H.N. (1973) Phytochemistry 12, 961—973
3 Huault, C., Faye, L., Klein-Eude, D., Fourcroy, P., Blondel, J.D. and Rollin, P. (1976) in Etudes de Biologie Végétale (Jacques, R., ed.), Edition du Phytotron, Paris
4 Havir, E.A. and Hanson, K.R. (1973) Biochemistry 12, 1583—1591
5 Nari, J., Mouttet, Ch., Pinna, M.H. and Ricard, J. (1972) FEBS Lett. 23, 220—224
6 Zimmermann, A. and Hahlbrock, K. (1975) Arch. Biochem. Biophys. 166, 54—62
7 Zucker, M. (1965) Plant Physiol. 40, 779—784
8 Walton, D.C. and Sondheimer, E. (1968) Plant Physiol. 43, 467—469
9 Minamikawa, T. and Uritani, I. (1964) Arch. Biochem. Biophys. 108, 573—574
10 Fritig, B., Gosse, J., Legrand, M. and Hirth, L. (1973) Virology 55, 371—379
11 Jangaard, N.O. (1974) Phytochemistry 13, 1769—1775
12 Engelsma, G. and Van Bruggen, J.M.H. (1971) Plant Physiol. 48, 94—96
13 Durst, F. and Mohr, H. (1966) Naturwissenschaften 24, 707—708
14 Attridge, T.H. and Smith, H. (1967) Biochim. Biophys. Acta 148, 805—807
15 Huault, C., Klein-Eude, D., Rollin, P. and Blondel, J.D. (1971) C.R. Acad. Sc. (Paris) 273, 745—748
16 Attridge, T.H., Johnson, C.B. and Smith, H. (1974) Biochim. Biophys. Acta 343, 440—451

17 Acton, G.J. and Schopfer, P. (1975) Biochim. Biophys. Acta 404, 231—242
18 Tong, W.F. and Schopfer, P. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 4017—4021
19 Johnson, C.B. and Smith, H. (1978) Phytochemistry 17, 667—670
20 Johnson, C.B. (1977) in Regulation of Enzyme Synthesis and Activity in Higher Plants (Smith, H., ed.), pp. 225—243, Academic Press, London
21 Hu, A.S.L., Bock, R.M. and Halvorson, H.O. (1962) Anal. Biochem. 4, 489—504
22 Fourcroy, P. (1978) Biochem. Biophys. Res. Commun. 84, 713—720
23 Blondel, J.D., Huault, C., Faye, L., Rollin, P. and Cohen, P. (1973) FEBS Lett. 36, 239—244
24 Malcoste, R., Huault, C., Larcher, G. and Rollin, P. (1972) Physiol. Veg. 10, 575—587
25 Vinograd, J. and Hearst, J.E. (1962) Fortschr. Chem. Org. Naturstoffe 20, 372—378
26 Attridge, T.H. and Smith, H. (1973) Phytochemistry 12, 1569—1574
27 Axen, R., Porath, J. and Erntack, S. (1967) Nature 214, 1302—1304
28 Maurer, H.R. (1971) in Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis, p. 46, W. de Gruyter, Berlin
29 Lin, A.W.S.M. and Castell, D.O. (1978) Anal. Biochem. 86, 345—356
30 Havir, E.A. and Hanson, K.R. (1968) Biochemistry 7, 1896—1903
31 Marsh, H.V., Havir, E.A. and Hansen, K.R. (1968) Biochemistry 7, 1915—1918
32 Schopfer, P. (1971) Planta 99, 339—346
33 Parkhust, J.R. and Hodgins, D.S. (1972) Arch. Biochem. Biophys. 152, 597—605
34 Kalghatgi, K.K. and Subba Rao, P.V. (1975) Biochem. J. 149, 65—72
35 Sizer, I.W. (1943) Adv. Enzymol. 3, 35—62
36 Hanson, K.R. and Havir, E.A. (1970) Arch. Biochem. Biophys. 141, 1—17
37 Datta, P. and Gest, H. (1965) J. Biol. Chem. 240, 3023—3033
38 Atkinson, D.E., Hathaway, J.H. and Smith, C. (1965) J. Biol. Chem. 240, 2682—2690
39 Nishikawa, A.H. and Bailon, P. (1975) Arch. Biochem. Biophys. 168, 576—584
40 Ochoa, J.L. (1978) Biochimie 60, 1—15
41 Tanaka, Y. and Uritani, I. (1977) J. Biochem. 81, 963—970
42 Levitzki, A. and Koshland, D.E. (1969) Proc. Natl. Acad. Sci. U.S. 62, 1121—1128
43 Levitzki, A. and Koshland, D.E. (1976) in Current Topics in Cellular Regulation (Horecker, B.L. and Stadtman, E.R., eds.), Vol. 10, pp. 1—40, Academic Press, New York
44 Gupta, S. and Acton, G.J. (1979) Biochim. Biophys. Acta 570, 187—197
45 Loomis, W.D. and Battaile, J. (1966) Phytochemistry 5, 423—438
46 Mohr, H. (1972) in Lectures on Photomorphogenesis, pp. 141—148, Springer Verlag, Berlin
47 Lamb, C.J., Merritt, T.K. and Butt, V.S. (1979) Biochim. Biophys. Acta 582, 196—212
48 Katz, J.J. and Crespi, H.L. (1970) in Isotope Effects in Reaction Rates (Collins, C.J. and Bowman, N.S., eds.), pp. 286—363, Van Nostrand-Reinhold Book Co, New York
49 Siegel, S.M. and Galun, E. (1978) Plant Cell Physiol. 19, 851—856
50 Moore, P.B. (1977) Anal. Biochem. 82, 101—108