EVIDENCE FOR ACTIVE AND INACTIVE FORMS OF L-PHENYLALANINE AMMONIA-LYASE IN ETIOLATED AND LIGHT-GROWN RADISH COTYLEDONS

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1. Introduction

L-Phenylalanine ammonia-lyase (EC 4.3.1.5) is an enzyme involved in the deamination of L-phenylalanine in *trans*-cinnamic acid. This is the first reaction of the very important biosynthetic pathways of phenolic compounds in higher plants [1]. This enzyme is one example of a set of enzymes controlled by phytochrome P_{fr}** [2]. In the dark-grown seedlings no phenylalanine ammonia-lyase activity (or extremely low levels) can be detected in crude extracts of the cotyledons. Exposure of cotyledons to wavelengths maintaining sufficient amounts of P_{fr} (710–720 nm) are responsible for the highest increases in phenylalanine ammonia-lyase activity [2, 3].

The observed effects of light can either reflect a de novo synthesis of active enzyme or the activation of a possible inactive precursor already present in dark-grown cotyledons [1]. Recently, in our laboratory, Klein-Eude et al. [4], from the results of experiments conducted using cycloheximide as protein synthesis inhibitor, postulated the existence of an inactive form of the enzyme in dark-grown cotyledons.

We have investigated this hypothesis using protein chemistry techniques. We present here experimental evidence for the presence, in etiolated cotyledons, of an enzymatically inactive protein species. Physico–chemical analysis of this protein indicates obvious analogies with the active enzyme purified from light-grown cotyledons.

2. Materials and methods

Cotyledons from light and dark-grown radish (*Raphanus sativus* cultivar 'longue rave saumonée') seedlings were cut off according to Klein-Eude et al. [4]. Handling of the organs was as described previously [4]. Extraction was performed with 120 ml of 20 mM ammonium acetate buffer, pH 6, on 25 g fresh cotyledons during 15 min at 0°C. The protein solution was recovered after centrifugation for 30 min at 30 000 *g*, filtered on glass wool, and then applied on a Sephadex G-25 column equilibrated in the same buffer.

All the proteins containing fractions (a total of 160 ml) recovered from the molecular sieve filtration were submitted to affinity chromatography on a Sepharose 4 B-phenylalanine conjugate. This was prepared from a 100 ml decanted Sepharose 4 B activated at pH 11 with 50 g BrCN. The gel was reacted

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** P_{fr}: active form of phytochrome absorbing far-red light.

with 400 ml of a L-phenylalanine 0.33 M solution at pH 9.5 for 24 hr at 5°C then transferred on a column, rinsed with 10 l distilled water in the cold. After the ammonium acetate solution of protein was applied at the top of the affinity column, this was abundantly washed with 5 l of the same buffer, The pH was then raised to 10.0 with ammonium hydroxide. Protein containing solutions were collected and freeze-dried for further analysis.

Isoelectric focusing was performed on a LKB 8 121, 110 ml, column (LKB Produkter). Handling of the column was essentially as described by Svensson [5]. A pH gradient 4–6 was obtained from commercial ampholytes (Ampholines LKB) at the concentration of 1%. A saccharose 0–50% (w/v) was used. 20 mg of proteins, recovered from the ammonium hydroxide eluted peak in the affinity chromatography, were submitted to focusing at 5°C during 36 hr under an applied tension of 500 V. Recovery of the gradient, at the end of the experiment, was done as described in [6] and pH values (Taccusel Minisis type), enzyme assay [4], protein content [7], were measured on each 1 ml fraction.

Polyacrylamide gel electrophoresis was carried out on 7.5% acrylamide gels according to the general technique of Davis [8] at various pH's (pH 3.5 buffered with 0.05 M formate, pH 9.2 buffered with 0.8 M Tris–EDTA) revealed by either Comassie Blue or Amido Black.

Sedimentation velocity coefficient was measured at 20°C with an Analytical Beckman model E ultracentrifuge, equipped with a Schlieren optics at 60 000 rpm. Protein concentration was 4 mg/ml in 0.05 M borate, pH 8.8 buffer. Molecular sieving was done in columns of Sephadex G-200 and Sepharose 4 B gels in 0.05 M borate buffer at pH 8.8.

Equilibrium dialysis was performed in 500 μl polymethacrylate cells using pretreated Visking Tubing 18/32. Handling of the samples and aliquots measurements were done as described in [9]. A 1–4 μM solution of protein was used and binding of the amino acid was measured over a concentration range 9 μM–150 μM with [^{14}C]phenylalanine (Service des Molécules Marquées. C.E.N. Saclay) at room temperature. Results were plotted according to Scatchard from measurements of bound and free concentrations of the ligand at equilibrium [9]. Radioactivity was evaluated by liquid scintillation using Bray's solution and an Intertechnique SL 40 spectrometer [9]. Antiserum against the active enzyme was prepared by injecting one rabbit with a highly purified prepararion of phenylalanine ammonia lyase obtained after affinity chromatography. Immunoelectrophoresis was performed according to Grabar and Williams [10].

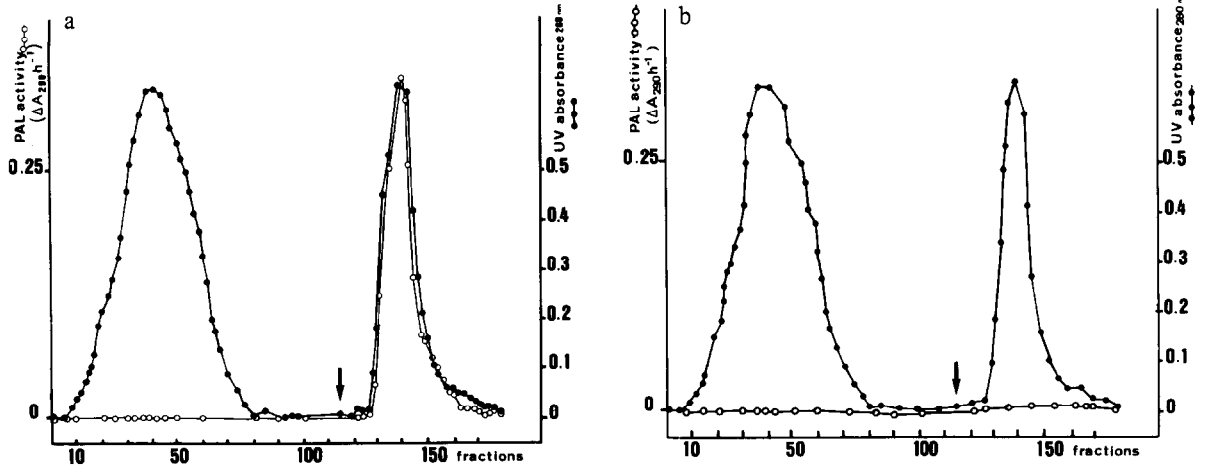

Fig. 1. Affinity chromatography of cotyledons' extracts on a phenylalanine–Sepharose 4 B conjugate. 500 mg of protein extracted from ligth (a) or dark-grown (b) cotyledons were applied on the column. The affinity column was washed with 20 mM ammonium acetate buffer, pH 6; protein contents (●——●——●) and enzyme activity (○——○——○) were measured on each 8 ml fraction. pH was changed from 6.0–10.0 (arrow) and elution performed with ammonium hydroxide solution.

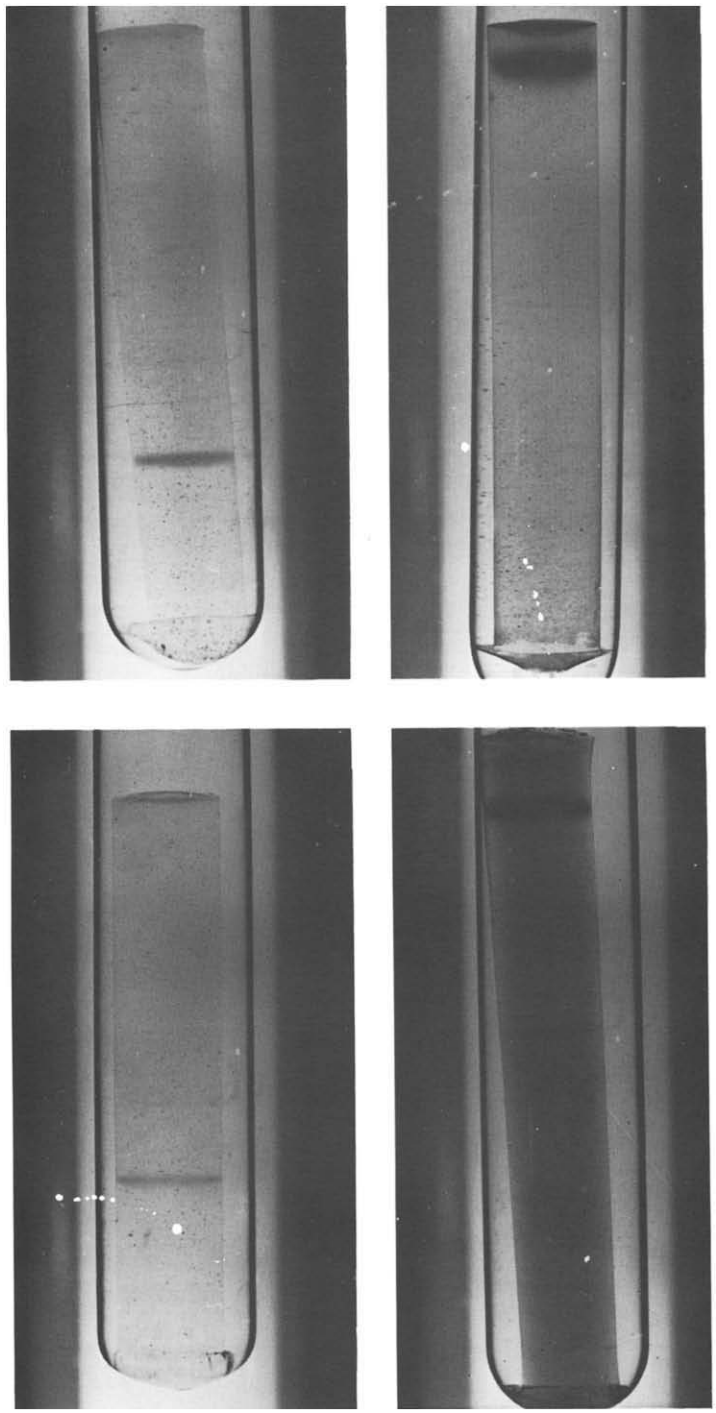

Fig. 2. Polyacrylamide gel electrophoresis of 'active' and 'inactive' forms of phenylalanine ammonia-lyase on 75% polyacrylamide gels. Migration is to the bottom figure: a) active form in 50 mM formate, pH 3.5; b) active form in 0.8 M Tris–EDTA, pH 9.2; c) inactive form in 50 mM formate, pH 3.5; d) inactive form in 0.8 mM Tris–EDTA, pH 9.2.

3. Results

3.1. *Purification and characterization of phenylalanine ammonia-lyase from light-grown cotyledons*

After extraction, then filtration on a Sephadex G-25 column, the crude mixture of proteins obtained from fresh cotyledons was submitted to affinity chromatography. A column of phenylalanine covalently bound to Sepharose 4 B was used. Adsorption was performed at pH 6.0. Sixty to seventy % of the total amount of proteins applied at the top of the affinity column was recovered in the void volume and found devoid of phenylalanine ammonia-lyase activity. When pH was increased to 10.0 a sharp peak of protein was eluted containing enzyme activity (fig. 1a). Control experiments were run using Sepharose 4 B. In the absence of ligand covalently bound on the agarose all the proteins and enzyme activity were eluted in the void volume at pH 6.0. The purified enzyme was then analyzed by different methods for purity controls. Polyacrylamide gel electrophoresis in different buffers indicated perfect homogeneity (fig. 2). Under pH 5.0 migration towards the cathode suggested an anionic protein. The enzyme sample recovered after desorption from the affinity adsorbent was submitted to isoelectric focusing in a pH and density gradient. Fig. 3a shows the diagram obtained when a pH range 4.0–6.0 was used. All the enzyme activity was found associated with the unique observable peak of protein at pH 4.9 ± 0.1. This, unambiguously, indicated the absence of any impurity in the compound exhibiting a strong affinity for the phenylalanine–Sepharose conjugate at pH 6.0.

Further analysis of the protein confirmed its perfect homogeneity. Upon Sepharose 4 B and Sephadex G-200 filtrations, elution patterns showed superposition of protein and enzyme activity peaks. Calculations done using molecular sieving behaviour (Sephadex G-200) and the equation of Marrink [11] led to a value of 2.8×10^5 daltons for the molecular weight. This result is in good agreement with reports in the literature [12–14] evaluating the molecular weight of phenylalanine ammonia-lyase from various plant species. $s^0_{20,w}$ Measured at pH 8.8 in 0.05 M borate was 12.18 ± 0.30 S.

3.2. *Purification of an inactive protein related to phenylalanine ammonia-lyase*

Extraction of the proteins from etiolated cotyledons was performed under similar conditions. Affinity chromatography of the crude material recovered after Sephadex G-25 filtrations led to the elution pattern of fig. 1b. Again there were comparable amounts of protein eluted at pH 6.0 and in the second peak at pH 10.0. But in this case no phenylalanine ammonia-

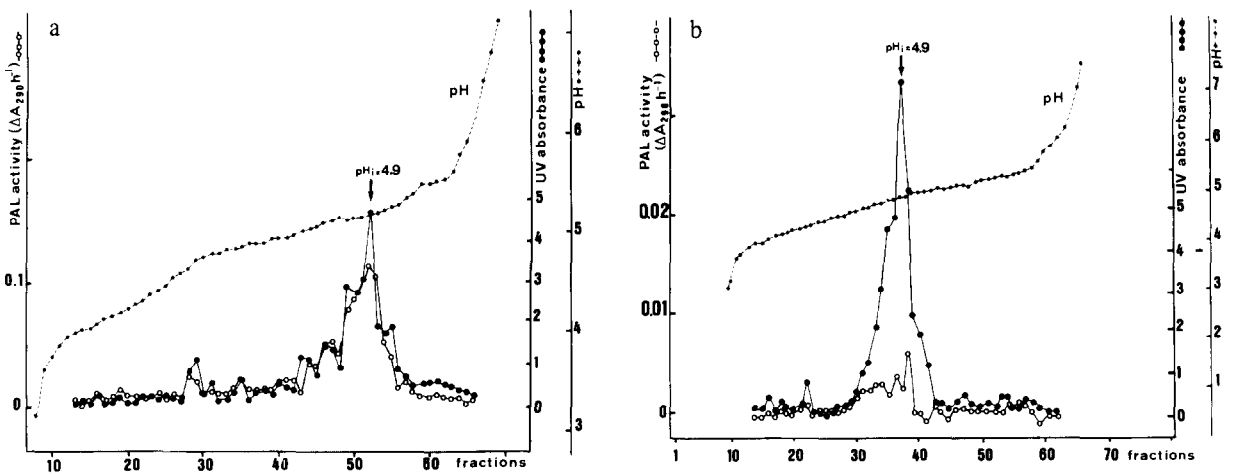

Fig. 3. Isoelectric focusing of enzymatically 'active' (a) and 'inactive' (b) proteins recovered from the affintiy chromatography. Elution diagram obtained after electrofocusing of 20 mg proteins during 36 hr at 5°C in a 110 ml LKB column. pH gradient from 4.0–6.0 in a sucrose (0–50% at 280 nm; w/v) gradient. Fraction volume = 1 ml./pH, UV absorbance and enzyme activity were measured on each fraction.

lyase activity could be detected in both fractions. The proteins eluted after change of pH from 6.0–10.0 was subjected to a systematical analysis by techniques identical to those used in the case of the active enzyme. Disc gel electrophoresis was indicative of a high degree of homogeneity (fig. 2). Isoelectric pH, evaluated by the isoelectric focusing technique, was there again 4.9 and no impurity was detected over the pH range used in the gradient (fig. 3b). The sedimentation velocity measurement ($s^0_{20,w}$ = 12.13 ± 0.70) confirmed the strong analogies between those proteins purified from dark- and light-grown cotyledons. The behaviour of the inactive protein upon Sepharose 4 B and Sepharose G-200 filtrations, was strictly similar to the one described above and indicated homogeneity. The previously described methods for molecular weight evaluation gave a value close to 2.8×10^5 daltons.

3.3. *Binding of L-phenylalanine in vitro*

Since the protein purified from extracts of etiolated cotyledons was adsorbed on the affinity column it was interesting to check its ability to bind non covalently L-phenylalanine. This was evaluated by equilibrium dialysis using the ^{14}C-labeled amino acid. In fig. 4 it can be seen that representation of the binding data according to Scatchard led to a linear isotherm over the range of ligand concentrations used. It is deduced from this experiment (fig. 4) that, at equilibrium, two molecules of L-phenylalanine can be bound per molecule of protein (assuming a mol. wt of 3×10^5) with an association constant of 0.57×10^5 M^{-1}.

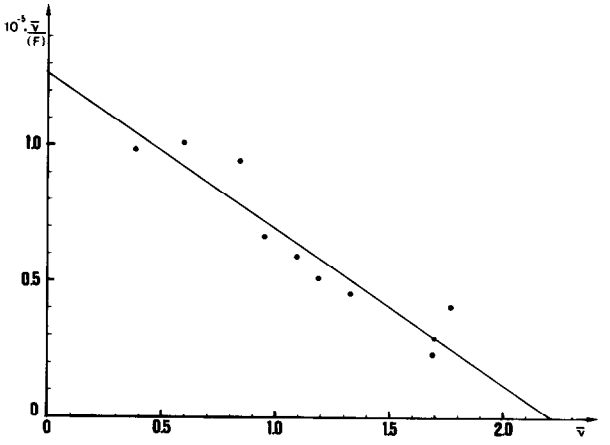

Fig. 4. Interaction of L-phenylalanine with the enzymatically inactive protein isolated from etiolated cotyledons. Binding data are expressed according to Scatchard. Protein (1 μM) and ligand (9–150 μM) solutions were in 20 mM ammonium acetate, pH 6.0. Dialysis time was on average 3 hr at room temperature. Each plotted value is an average of three aliquot measurements at each ligand concentration run in quadruplicate.

3.4. *Immunoreactivity of both active and inactive forms of phenylalanine ammonia-lyase*

Immunoelectrophoresis was used to compare active and inactive forms of the enzyme. In fig. 5 it can be seen that both proteins react with the antiserum prepared from the active enzyme. The pattern observed indicates complete antigenic identity.

4. Discussion

The affinity chromatography technique developed here appears to be adequate for 'fishing out', from the light-grown cotyledons' proteins extracts, phenylalanine ammonia-lyase. Comparison with the physico–chemical properties of the proteic species isolated from etiolated cotyledons by an identical method suggests strong homologies. The presence in those dark-grown organs of a compound exhibiting a strong affinity for the normal enzyme substrate, both free and covalently bound to agarose, suggests that the structural basis for phenylalanine recognition is preserved in this protein. The finding that, *in vitro*, two phenylalanine molecules can be bound per molecule of inactive protein is in good accord with the recent report by Havir and Hanson [15] that the active tetrameric enzyme from maize possesses two catalytic sites. This can also be correlated with similar results from Ricard et al.

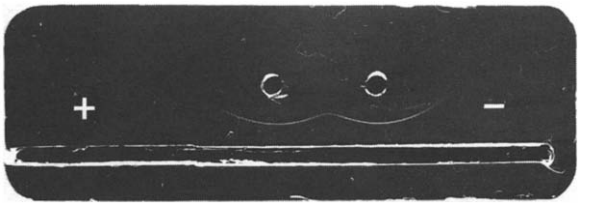

Fig. 5. Immunoreactivity of both active and inactive forms of L-phenylalanine ammonia-lyase. Immunoelectrophoresis was as described in Materials and methods. Antigens wells were respectively filled with active (right) and inactive (left) protein solutions. Antiserum was placed in the trough.

(personal communication) obtained with the enzyme from wheat. Photoregulation of this enzyme activity and, possibly, conversion from the 'inactive' to the 'active' form of the protein must imply discrete structural rearrangements. Those modifications that determine catalytic activity are not as yet known. It is interesting to note, in this respect, that immunoelectrophoresis performed with antibodies prepared from 'active' phenylalanine ammonia-lyase indicate complete antigenic identity.

It is difficult, at the moment, to exclude the possibility that the so-called 'active' enzyme extracted after far-red light exposure of the cotyledons is a heterogeneous population of both activated and inactive 'precursor'. Specific activity of this fraction was found to increase linearly with duration of the 710–720 nm irradiation of the cotyledons [16]. The observation that the total amount of affinity adsorbed protein was not significantly increased upon irradiation, might support this hypothesis.

Phytochrome dependent molecular processes involved in the photoconversion of this protein are now under investigation in these laboratories.

Acknowledgements

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References

[1] Smith, H.. (1972) in: Phytochrome (Mitrakos, K. and Shropshire, W. Jr., eds.) p. 433, Academic Press, London.
[2] Mohr, H. (1972) Lectures on photomorphogenesis, Springer-Verlag Berlin.
[3] Huault, C., Klein-Eude, D., Rollin, P. and Blondel, J.D. (1971) Compt. Rend. 273, 745.
[4] Klein-Eude, D., Rollin, P. and Huault, C. (1973) Plant Science Letters, in press.
[5] Svensson, H. (1962) Arch. Biochem. Biophys. Suppl. I, 132.
[6] LKB 8 100 Ampholine (Electrofocusing Equipment) Instruction Manual S-161 25 Bromma 1, Sweden.
[7] Lowry, O.H., Rosebrough, W.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.
[8] Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404.
[9] Camier, M., Alazard, R., Cohen, P., Pradelles, P., Morgat, J.L. and Fromageot, P. (1973) European J. Biochem. 32, 207.
[10] Grabar, P. and Williams, C.A. (1953) Biochim. Biophys. Acta 10, 193.
[11] Marrink, J. and Gruber, M. (1969) FEBS Letters 2, 242.
[12] Nari, J., Mouttet, Ch., Pinna, M.H. and Ricard, J. (1972) FEBS Letters 23, 220.
[13] Hodgins, D.S. (1971) J. Biol. Chem. 246, 2977.
[14] Havir, E.A. and Hanson, K.R. (1968) Biochemistry 7, 1896.
[15] Havir, E.A. and Hanson, K.R. (1973) Biochemistry 12, 1583.
[16] Huault, C., in preparation.