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SUB-CELLULAR DISTRIBUTION OF ALANINE AMINOTRANSFERASE IN LEAVES OF *LOLIUM TEMULENTUM*

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Abstract—Chloroplasts isolated from leaves of *Lolium temulentum* by differential centrifugation and sucrose gradient centrifugation were found to contain high levels of GPT activity. A plastid form of the enzyme could be separated from a cytoplasmic isozyme by DEAE-cellulose chromatography. Non-green tissue such as roots and dark-grown leaves contained negligible amounts of the plastid isozyme. During the greening of etiolated leaves the amounts of both forms of the enzyme increased, the plastid isozyme at a higher rate than the cytoplasmic isozyme.

INTRODUCTION

IT IS well established that animal cells contain at least two isozymes of alanine aminotransferase (glutamate–pyruvate transaminase; GPT, E.C.2.6.1.2), one of which is localized in the mitochondrion and the other in the cytoplasm.[1] Similarly, a distinct form of GPT occurs in the mitochondria of peas (Kollöffel, personal communication). There are, however, conflicting reports concerning the existence of GPT in the chloroplast. Santarius and Stocking[2] found that as much as 58% of the total GPT activity of *Phaseolus* leaves was located in the plastids. Moreover, a number of workers, notably Kasparek[3] and Kirk and Leech[4] have detected several transaminase activities, including GPT, in plastids. On the other hand Hatch and Mau[5] were unable to find a plastid-associated form of GPT in leaf tissue of plants photosynthesizing by the C_4 pathway. Previous reports from this laboratory[6–8] described variations in the GPT activity of *Lolium temulentum* leaves in response to treatment with light and growth regulators. GPT activity declines as the leaf passes into the senescence phase of development. Treatments that tend to promote senescence, such as long days or gibberellic acid, also promote the decline in enzyme activity, while short days or inhibitors of gibberellin biosynthesis delay the onset of both senescence and GPT decay. Furthermore, during the course of the development of light-grown leaves and during the greening of dark-grown leaves on exposure to light, there is a close correlation between GPT activity and chlorophyll content. These results point to the existence in *L.*

[1] WILKINSON, J. H. (1970) *Isoenzymes*, Chapman & Hall, London.
[2] SANTARIUS, K. A. and STOCKING, C. R. (1969) *Z. Naturforsch.* **24b**, 1170.
[3] KASPAREK, M. (1968) *Physiol. Veg.* **6**, 19.
[4] KIRK, P. R. and LEECH, R. M. (1972) *Plant Physiol.* **50**, 228.
[5] HATCH, M. D. and MAU, S.-L. (1973) *Arch. Biochem. Biophys.* **156**, 195.
[6] HEDLEY, C. L. and STODDART, J. L. (1971) *J. Exp. Botany* **22**, 239.
[7] HEDLEY, C. L. and STODDART, J. L. (1971) *J. Exp. Botany* **22**, 249.
[8] HEDLEY, C. L. and STODDART, J. L. (1971) *Planta* **100**, 309.

temulentum of a plastid form of GPT. In the present paper we report some results from an investigation of the sub-cellular distribution of GPT activity in leaves of *L. temulentum*.

RESULTS

Hedley and Stoddart[8] showed that GPT activity in *Lolium* was low in emerging leaves and in the leaves of dark-grown seedlings, rose to a maximum in mature leaves grown in the light and decreased rapidly prior to or during senescence. The GPT activity of cell fractions prepared by differential centrifugation was determined using leaves of dark-grown seedlings and mature green or senescent material from light-grown plants.

Figure 1 shows the relative distribution of enzyme activity between the 1000 *g* pellet, composed predominantly of plastids, and the 20000 *g* fraction which was shown by electron microscopic examination to contain both mitochondria and microbodies. The amount of soluble activity remaining in the supernatant is also indicated. It was found that between 55 and 75% of the total recoverable activity was located in the supernatant fraction at each of the three stages of development. In dark-grown and mature green tissue the 1000 *g* pellet contained most of the remaining activity. However, in senescent leaves, the GPT activity in the plastid fraction was very low in comparison to the activity in each of the other fractions.

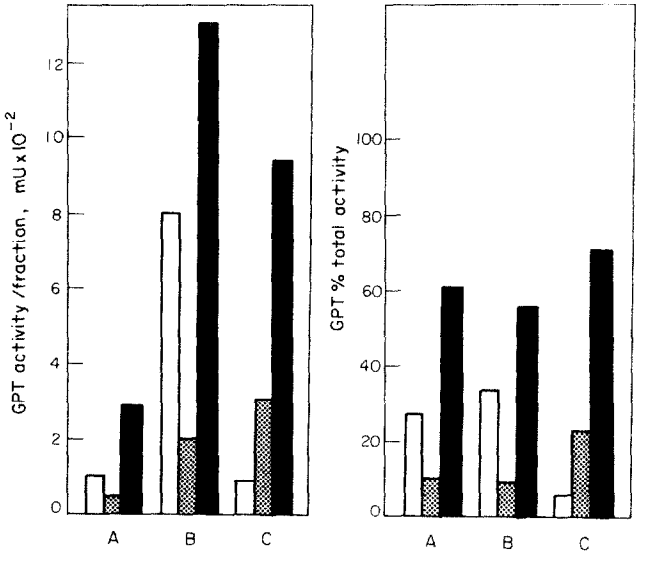

FIG. 1. GPT CONTENT OF SUBCELLULAR FRACTIONS FROM: (A) DARK-GROWN, (B) MATURE, AND (C) SENESCENT LEAVES OF *Lolium temulentum*, EXPRESSED ON THE BASIS BOTH OF TOTAL ACTIVITY AND RELATIVE ACTIVITY.

It is evident that at least 25–45% of the GPT activity in *Lolium* leaves is pelletable and, consequently, associated with sub-cellular particles. A particulate distribution of GPT can also be demonstrated by fractionating leaf organelles on sucrose density gradients and determining the enzyme distribution after equilibrium conditions have been established.[9] Figure 2 gives an example of a density gradient fractionation of a crude homogenate of mature green *Lolium* leaves.

[9] HUANG, A. H. C. and BEEVERS, H. (1971) *Plant Physiol.* **48**, 637.

GPT activity was found in four main regions of the gradient but most of the activity was located at the top (fractions 1–6) and this represented soluble enzyme plus activity leached from particulate matter during extraction and centrifugation. The activity in frac-

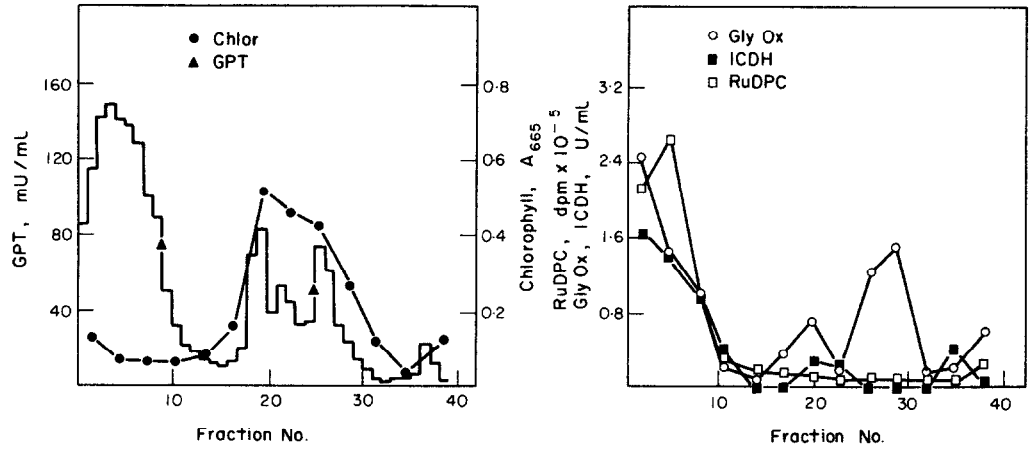

FIG. 2. DISTRIBUTION, ON A SUCROSE DENSITY GRADIENT, OF PARTICULATE ENZYMES FROM A HOMOGENATE OF *Lolium temulentum* LEAVES.

tions 7–13 was thought to be associated with mitochondria and the distribution of the marker enzyme, isocitrate dehydrogenase (ICDH), suggested that these organelles only partially entered the gradient. Within the gradient there were three distinct peaks of activity, all coinciding with the plastid region as marked by chlorophyll banding. The upper (lightest) zone corresponded with plastid fragments having a density of 1·195, whilst the lower bands (densities 1·215 and 1·235) represented two classes of intact plastids. The composition of the plastid bands was determined by examination under the light microscope and here it was clear that most of the intact plastids had been stripped of their outer membranes during isolation. No obvious structural distinction could be drawn between the material recovered from the two heavier bands but it may be that these represented chloroplast aggregates of varying sizes.

The distribution of the microbody marker enzyme, glycollate oxidase, was also determined and the major peak was found to have a density of 1·25 which closely approximates to that reported for microbodies from other plant tissues.[9] This density is higher than those determined for the GPT peaks. A smaller zone of glycollate oxidase was, however, associated with the plastid fragment band and some ICDH activity was also detected in this region. This may possibly be due to the trapping of mitochondria and microbodies, an effect already reported for wheat organelles by Feierabend and Beevers.[10]

These results indicate that a large proportion of the extractable GPT in *Lolium* leaves is associated with the plastid fraction but studies on the subcellular distribution of enzymes

[10] FEIERABEND, J. and BEEVERS, H. (1972) *Plant Physiol.* **49**, 33.

carried out by these methods are of limited quantitative accuracy. This is due to the inevitable damage occurring to the organelles and to the consequent release of compartmentalised activity into the soluble phase. For example, Fig. 2 shows that the plastid-based enzyme ribulose-1,5-diphosphate carboxylase (RuDPC) was almost completely lost into the soluble fraction during the isolation and fractionation procedures.

In order to quantitate the contribution of plastid GPT towards the total activity of the leaf a different approach had to be used. Ziegenbein[11] separated GPT from rat heart into two isozymes by DEAE-cellulose chromatography and showed that one form was localized in the mitochondria and the other in the cytoplasm. Similar experiments were carried out with *Lolium* leaf GPT. The distribution of GPT activity was determined over the elution profile from a DEAE-cellulose column loaded with a crude soluble enzyme extract from mature leaves of *L. temulentum*. Two forms of the enzyme were detected: a non-binding species emerging with the void volume and a second form which was eluted with a gradient of increasing NaCl concentration. The pattern could not be altered significantly either by reducing the ionic concentration of the equilibration buffer or by chromatography on QAE-Sephadex, a stronger binding ion-exchange material. The enzyme activity in both the bound and unbound fractions was destroyed by boiling. It is concluded, therefore, that both peaks were isozymes of GPT and that neither is due to endogenous keto-acids, which would give a positive reaction in the colorimetric assay used to detect the enzyme. A useful degree of purification was achieved during DEAE-cellulose chromatography. Typical figures for specific activities before and after this step are: total extract 4·2 mU/mg protein; unbound enzyme 76·9 mU/mg protein; bound enzyme 30·9 mU/mg protein.

In order to determine whether either of the GPT isozymes was localized in the chloroplasts, a 1000*g* pellet fraction from mature leaf homogenate was lysed by sonication in Tris-GSH buffer and, after centrifugation to remove plastid fragments, the extract was chromatographed on DEAE-cellulose. Only the non-binding form of GPT was present in the elution profile and this strongly suggests that the binding form is located outside the plastid. Further confirmation was the observation that *Lolium* root tissue contains only the bound form of the enzyme and this also suggests a relationship between the unbound species and the photosynthetic activities of the leaf. Thus, there are good indications that DEAE-cellulose chromatography gives a quantitative picture of the relative contributions of the plastid and the cytoplasm towards the total GPT activity of leaf tissue.

Chromatography on DEAE-cellulose was employed to investigate the appearance of GPT during the greening of etiolated seedlings. Chlorophyll and GPT increase during the first 4 days after transfer into the light confirming the previously reported relationship.[8] DEAE-cellulose column separations of GPT isozymes during the same period showed that dark-grown leaves contain negligible amount of the non-binding isozyme. During the succeeding 4 days, both the bound and unbound forms increase in activity, but at disparate rates so that by the fourth day the amount of non-binding GPT exceeds that of the bound enzyme.

DISCUSSION

The present results show that a large proportion of the GPT of *Lolium* leaves is localized in the chloroplasts. A number of different roles for plastid-associated transaminases have been suggested. They may function in the biosynthesis of amino acids from photosynthetic intermediates.[4] Certain amino acids, including alanine, are rapidly labelled in leaves and

[11] ZIEGENBEIN, R. (1966) *Nature* **212**, 935.

algal cells photosynthesizing in the presence of $^{14}CO_2$; moreover, in *Vicia faba* plastids, glutamate is the most abundant amino acid and GPT and aspartate aminotransferase (glutamate oxaloacetate transaminase; GOT) are the highest transaminase activities. On the basis of these observations Kirk and Leech[4] suggest that glutamate, the product of the photoreductive amination of α-oxoglutarate by glutamic dehydrogenase,[12] is the source of the amino group for the biosynthesis of other plastid amino acids. This scheme raises questions about the precise localization of GPT since the source of glutamate is the plastid whereas the co-substrate, pyruvate, comes from outside the plastid. Figure 2 shows a high level of GPT activity associated with *Lolium* plastids. On the other hand practically all of the plastid enzyme RuDPC has been released into the soluble fraction. On this evidence, GPT and RuDPC would appear to differ in the nature of their association with the plastid. GPT may be bound in some way—to a plastid membrane, possibly—since chloroplast fragments fractionated by sucrose gradient centrifugation retain some GPT activity.

An alternative role for plastid-associated GPT relates to the specificity of plant transaminases. As Matherton and Moore [13] have pointed out, amino acid and keto acid specificities have been established for few plant transaminases. The same workers have described a partially-purified enzyme from pea seedlings that transaminates a range of aromatic and aliphatic amino acids with α-oxoglutarate, pyruvate or oxaloacetate as co-substrate. It may be, therefore, that the enzyme assayed as GPT in the present experiments catalyzes a different transamination *in vivo*. Hedley and Stoddart[8] have suggested that the *in vivo* enzyme might use α-oxoglutaraldehyde as the co-substrate with alanine to produce δ-aminolaevulinic acid, an intermediate in chlorophyll biosynthesis. The correlation between GPT activity and the chlorophyll content of *Lolium* leaves observed by Hedley and Stoddart[7,8] is consistent with a postulated function for the enzyme in chlorophyll biosynthesis.

Andrews *et al*[14] and Hatch and Mau[5] have proposed a role for GPT and GOT in the C_4 pathway of photosynthesis. C_4 Plants contain as much as 20 times the level of GPT and GOT found in C_3 plants. Bundle sheath cells and mesophyll cells of C_4 plants contain different isozymes of GPT and of GOT. There is evidence that the keto acid products of photosynthesis are transaminated and shuttled between the two tissues as alanine and aspartate. The results described in the present paper contrast with those of Hatch[15] and Hatch and Mau,[5] who were unable to detect a particulate form of GPT in C_4 species. Furthermore the GPT isozymes of C_4 species are readily separated by gel electrophoresis,[5] unlike the GPT isozymes of *Lolium* which remain unresolved under a range of elecrophoretic conditions (Thomas, unpublished). Thus the distribution, properties and (possibly) functions of the GPT isozymes of *Lolium*, a C_3 plant,[16] are quite different from those of GPT from C_4 species. It remains to be seen whether it is generally true that the transaminases of C_3 and C_4 plants differ in this way.

The existence of different forms of GPT inside and outside the chloroplast raises questions about the synthesis of the different isozymes. Hedley and Stoddart[8] found that the increase in GPT activity on exposing the leaves of dark-grown *Lolium* seedlings to light was inhibited by cycloheximide but chloramphenicol, at a concentration that depressed the synthesis of chlorophyll and Fraction 1 protein, stimulated GPT. This indicates that

[12] Givan, C. V., Givan, N. A. and Leech, R. M. (1970) *Plant Physiol.* **45**, 624.
[13] Matherton, M. E. and Moore, T. C. (1973) *Plant Physiol.* **52**, 63.
[14] Andrews, T. J., Johnson, H. S., Slack, C. R. and Hatch, M. D. (1971) *Phytochemistry* **10**, 2005.
[15] Hatch, M. D. (1973) *Arch. Biochem. Biophys.* **156**, 207.
[16] Treharne, K. J. and Cooper, J. P. (1969) *J. Exp. Botany* **20**, 170.

GPT is made by the cycloheximide-sensitive 80*S* ribosomes of the cytoplasm and not by the 70*S* ribosomes of the plastid. Despite the fact that the site of synthesis of both isozymes appears to be the same, the activities of the two forms of the enzyme increase at different rates during illumination. A selective control of synthesis or turnover is indicated. The careful studies of Trewavas[17] have shown that plastid RNA and cytoplasmic RNA turn over at different rates. This is likely to be true for plastid and cytoplasmic enzymes. In this connexion it is interesting that GOT, tyrosine aminotransferase and glutamate dehydrogenase in *Tetrahymena* appear to be regulated partly or wholly by a system of degradative control.[18]

EXPERIMENTAL

Plant material. Lolium temulentum L. (Ba 3081) was used for all experiments. Plants were grown in a glasshouse without supplementary lighting or heating, in John Innes No. 1 potting compost. Enzyme preparations and subcellular fractionations were made from the youngest fully-expanded leaves and, in some experiments, from leaves showing obvious signs of senescence. Etiolated material was obtained from seedlings germinated and grown in continuous darkness at 20° in shallow trays of sand. After 7 days in darkness plants were transferred to the light (fluorescent source, intensity *ca* 32 lx). Roots were obtained from sand-grown seedlings exposed to light for 7 days.

Enzyme extraction. Soluble enzymes were extracted in Tris-HCl buffer, 0·05M, pH 7·5, containing 2 mM glutathione (GSH). Tissue was homogenized in buffer with sand in a chilled pestle and mortar and spun at 2000 *g* for 20 min. All operations were carried out at 2–4°.

Enzyme assays. GPT was assayed colorimetrically as described by Hedley and Stoddart.[6] Glycollate oxidase (E.C.1.1.3.1.) was assayed by the phenylhydrazine method of Dixon and Kornberg.[19] Isocitrate dehydrogenase (E.C.1.1.1.42) was determined by measuring $NADP^+$ reduction in the presence of isocitrate. Ribulose diphosphate carboxylase (RuDPC, E.C.4.1.1.39) was assayed radiochemically as described by Treharne and Cooper.[16] Chlorophyll was estimated by the method of MacKinney[20] as described by Hedley and Stoddart.[8]

Subcellular fractionation. All buffers contained Tris-HCl 0·05 M, $MgSO_4$ 0·01 M, EDTA 0·25 mM and GSH 2·0 mM at pH 7·5 (TMESH). The temperature was maintained at 2–4° during fractionation. Tissue was chopped finely and 5 *g* blended gently with 5 ml extraction buffer (TMESH containing 25% sucrose) in a pestle and mortar without sand. The mixture was filtered through two layers of "Miracloth" and the residue re-extracted by grinding briefly with a little sand and 5 ml extraction buffer. The combined filtrates were centrifuged to give a 1000 *g* pellet, a 20000 *g* pellet and a supernatant. Pellets were resuspended in TMESH prior to enzyme assay. Organelles were fractionated by sucrose density gradient centrifugation similar to the method of Huang and Beevers.[9] 15 ml gradients of 25–65% sucrose, TMESH over 1·5 ml 80% sucrose, TMESH were prepared in 20 ml polythene tubes and 4 ml tissue homogenate layered on top. Gradients were spun for 3 hr at 30000 rev/min (100000 *g* avg.) in the 3 × 23 ml swing-out rotor of an MSE super-speed 50 centrifuge. Assays were carried out on 0·6 ml fractions taken from the gradient by a Buchler Auto-densiflow and a BTL Chromafrac fraction collector. Chlorophyll was measured in terms of absorbance at 665 nm. Density values were estimated at specific points in the gradients by means of refractometry.

DEAE-cellulose chromatography. The crude soluble enzyme extract was chromatographed on a small (2·5 × 3·0 cm) column of DEAE-cellulose (Whatman DE52, preswollen) equilibrated with Tris-GSH extraction buffer. The column was eluted with 50 ml of a 0–0·4 M NaCl gradient at 36 ml/hr and 2·4 ml fractions were assayed for GPT activity.

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