Spatial and Temporal Influences on the Cell-Specific Distribution of Glycine Decarboxylase in Leaves of Wheat (Triticum aestivum L.) and Pea (Pisum sativum L.)

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ABSTRACT

The distribution of glycine decarboxylase (GDC) in leaves of pea (Pisum sativum L.) and wheat (Triticum aestivum L.) has been investigated using immunogold labeling of the P-protein subunit of the GDC complex. Mitochondria in photosynthetic mesophyll cells were densely labeled, whereas those in nonphotosynthetic vascular parenchyma and epidermal cells were only weakly labeled. In pea leaves the density of immunogold labeling on mitochondria in the chloroplast-containing bundle sheath and stomatal guard cells was intermediate between that in mesophyll and epidermal cells. In both species the density of labeling on mitochondria in a cell appeared to reflect the photosynthetic capacity of the cell. This relationship was further examined in wheat where a natural developmental axis exists along the lamina such that cell maturity increases with distance from the basal meristem. In this case the density of labeling on mesophyll cell mitochondria increased with photosynthetic development and with increasing maturity of the cell. Vascular cell mitochondria, however, became less densely labeled as the cells matured. The results indicate a close, positive correlation between the concentration of GDC in the mitochondria and the photosynthetic status of the host cell. This relationship is maintained effectively under the influence of both spatial (i.e. cellular differentiation across the lamina) and temporal (i.e. cellular development along the lamina) constraints.

Glycine decarboxylase (GDC) is a multienzyme complex consisting of four different subunits (P, H, T, and L) and is localized in the mitochondria (15, 22). The major function of the complex in higher plants is to oxidize glycine, an intermediate of the photosynthetic pathway. The substrate for photosynthesis, phosphoglycolate, originates from the oxygenase reaction of the chloroplast enzyme RuBP carboxylase and is therefore restricted to photosynthetically active tissue (17). This raises the question as to whether the GDC complex is similarly segregated. It has previously been shown that the capacity for glycine oxidation is severalfold higher in mitochondria isolated from photosynthetic organs than in those isolated from nonphotosynthetic tissues, e.g. roots and leaf veins, where activity is virtually undetectable (10). Similarly, in C4 plants, GDC activity is present only in those cells which contain RuBP carboxylase, the bundle sheath cells, and is absent from mesophyll cells (18). A recent study with C3-C4 intermediate species from four genera (13, 20) illustrates further complexity in that, although RuBP carboxylase is found in both mesophyll and bundle sheath cells, GDC is confined to the latter and this leads to more efficient recapture of photorespired CO2 (20).

Whereas there is evidence for cell-specific localization of GDC in higher plants, such studies have only addressed the distribution of GDC in relation to the photosynthetic mechanism used. The heterogeneous nature of leaf tissue is generally overlooked in biochemical studies. In C3 leaves such as wheat and pea, for example, only 50% of the cells are chloroplast-containing mesophyll cells (14) capable of photosynthesis. The remaining cells are predominantly vascular and, in young expanding leaves, still living (14) and must consequently contain mitochondria. The possibility therefore arises that these discrete populations of mitochondria may differ in their ability to metabolize glycine. In addition to this spatial separation of mitochondrial populations there also exists a developmental, or temporal, influence on leaf organelle metabolism. This is most readily studied in monocotyledonous leaves, such as wheat, where all of the cells are derived from divisions within a basal meristem. This produces a natural developmental system where the age of a cell and, more specifically, the photosynthetic activity of a mesophyll cell (16, 21) increases with its displacement away from the leaf base.

In the present study we have investigated the distribution of the P-protein of the GDC complex within leaf cells differing in both their specialization (photosynthetic and nonphotosynthetic) and in their maturity. The results have important implications in the interpretation of previous studies of respiratory metabolism in isolated mitochondria and in vivo.

MATERIALS AND METHODS

Growth of Plants

Pea seeds (Pisum sativum L. cv Birte) were imbibed in running tap water overnight and seedlings were grown in trays

1 Supported by The Royal Society (University Research Fellowship to A. K. T.) and the Agricultural and Food Research Council (grant no. PG85/500 to A. K. T.; grant-in-aid to S. R.).

2 Abbreviations: GDC, glycine decarboxylase; RuBP, ribulose bisphosphate.
of vermiculite in a naturally lit glasshouse at 20°C day and 16°C night temperatures. Plants were supplied with tap water as necessary, and fully expanded leaflets were harvested 15 d after sowing.

Wheat (Triticum aestivum L., cv Maris Huntsman) was grown in a controlled-environment cabinet as described in (21). Primary leaves were harvested at 8 d when their growth rate was at a maximum (21).

**Antiseras**

Immunoglobulin fraction of rabbit serum containing antibody raised against the 98-kD subunit (P-protein) of the glycine decarboxylase complex from pea leaves was kindly provided by Prof. D. J. Oliver (University of Idaho, Moscow, ID). Specificity was tested by Western blotting of crude pea and wheat leaf extracts following SDS-PAGE as described by Rawsthorne et al. (20).

**Preparation of Leaves for Electron Microscopy**

Sections (approximately 1.0 mm wide) of pea leaves were fixed and embedded as described by Rawsthorne et al. (20).

Sections (approximately 1.0 mm transverse) of wheat primary leaves were taken at 2.0, 4.0, 6.0, and 8.0 cm from the basal meristem and immediately immersed in a fixative solution containing 4% (v/v) formaldehyde, 0.5% (v/v) glutaraldehyde, and 0.2 M K-phosphate (pH 7.2). After 5 h in the fixative solution, the sections were washed overnight in 0.2 M K-phosphate (pH 7.2), dehydrated in an ethanol series, and then placed in LR gold resin (London Resin Company, Basingstoke, UK). The sections were infiltrated with resin on a rotator for 10 d with several changes of LR gold; during the last 2 to 3 d 0.1% (w/v) Benzil (the light-sensitive resin initiator) was added and the resin was light-polymerized according to the supplier's recommendations, except that polymerization was enhanced by increasing the light treatment to 48 h. The temperature was maintained at 4°C throughout.

**Immunolabeling of Tissue**

Thin sections of embedded pea leaves were immunolabeled as described by Rawsthorne et al. (20). The antibody to the P-protein subunit (referred to hereafter as GDC antibody) was used at a dilution of 1:300 (v/v). The size of the goat-anti-rabbit gold probe (Janssen Life Products) was 15 nm.

Thin sections were cut from the embedded wheat leaf tissue, picked up on 300 mesh nickel grids, and allowed to dry at room temperature. All incubations were performed by inverting the grids onto a 20 μL droplet of the incubation solution on Parafilm in a Petri dish. Washes were carried out by placing the grids in the rinsing solution in the wells of a microtiter plate. All rinses and dilutions of gold probe and antisera were made in PBS* (PBS containing 1% [w/v] BSA, 1 mM Na-EDTA, 0.05% [v/v] Tween-20 and 0.02% [w/v] NaN3). The grids were initially blocked by incubation for 30 min at room temperature in a 1:10 (v/v) dilution of goat serum and then incubated overnight at 4°C (in a humid box) in a 1:600 (v/v) dilution of the GDC antibody. After 5 × 2 min rinses in PBS* the grids were gold-labeled by incubating them for 1 h at room temperature with a 1:10 (v/v) dilution of 5 nm goat-anti-rabbit gold probe (BioCell Research Laboratories, Cardiff, UK). The grids were then thoroughly rinsed, first in PBS* (3 × 10 min) and then in double glass-distilled water (4 × 3 min) before being lightly poststained in saturated aqueous uranyl acetate for 3 min.

**Quantitative Distribution of Immunogold Particles**

**Pea**

The total number of 15-nm gold particles was counted within a defined area (i.e. mitochondria and background) on micrographs at ×35,000 magnification. At least 30 mitochondrial profiles were assessed from each of nine individual mesophyll, vascular (parenchyma), or epidermal cells on a single representative immunolabeled section of leaf tissue. Sixteen mitochondrial profiles were assessed from a pair of stomatal guard cells and from two individual bundle sheath cells. Total profile areas per cell were determined directly from the micrographs using a computer graphics tablet. Density of immunogold labeling was calculated by dividing the total counts in all of the mitochondria in a given cell by the total mitochondrial profile area per cell. Background was determined in the same way by counting the label within the remainder of the cell.

**Wheat**

Random micrographs of mitochondria (at ×26,000) within vascular and mesophyll cells were taken on a JEOL 100c electron microscope at 100 kV and printed at ×78,000 magnification. Counts of the number of 5-nm gold particles per individual mitochondrial profile were made for a total of 31 (mesophyll) or 52 (vascular parenchyma) mitochondria and after calculating profile areas, results were expressed as counts/μm². The number of gold particles in a random 0.25 μm² area of background (as above) was also counted for each of the mitochondrial profiles examined.

The procedural differences for the two species are due to the optimization of techniques in two different laboratories—the pea leaf work was carried out at Norwich (S. R. and C. M. H.) and the wheat at Sussex (A. K. T. and J. R. T.).

**Data Analysis**

Statistical analysis (mean, standard error and Student's t-test) was performed using MiniTab (MiniTab, Inc., PA).

**Determination of GDC Activity**

**Preparation of Leaf Protoplasts and Lysates**

Sections (5 mm transverse) were taken at 1.0 cm intervals between 3.0 and 8.0 cm from the basal meristems of 8-d-old wheat primary leaves. Protoplasts were prepared from each of these regions of the leaf by cutting the sections into 1 to 2 mm slices and incubating them for 3 h in a digestion medium containing 0.5 M sorbitol, 1 mM MgCl₂, 5 mM Mes (pH 6.0), 2% (w/v) Cellulase (Onozuka, supplied by Unwins, Welwyn, UK), and 0.2% (w/v) Pectolyase (Sigma, Poole, UK). Follow-
ing their purification on sucrose/sorbitol gradients (9) the protoplasts were resuspended in 0.5 M sorbitol, 1 mM CaCl₂, 5 mM KH₂PO₄, 5 mM MgCl₂, and 30 mM Hepes-NaOH (pH 7.0). The number of protoplasts in each preparation was determined by counting protoplasts in an aliquot using a 0.2 mm deep hemocytometer under a light microscope. This enabled the activity to be expressed relative to the number of mesophyll cells extracted, since protoplasts prepared in this way are all derived from mesophyll cells. Intactness of the protoplasts at this stage was virtually 100% as determined by examination under the microscope (Broken protoplasts lose their spherical shape and the chloroplasts become displaced from the perimeter of the plasma membrane.) Following this analysis the protoplasts were lysed by injecting them once through 20 μm nylon mesh attached to a 15 mL plastic syringe; this resulted in breaking all of the protoplasts but maintained the organelles intact (19). The lysate was used directly in the GDC assay.

GDC

GDC was assayed by a method developed from that of Walton and Woolhouse (23). The reaction mixture contained 0.5 M sorbitol, 1 mM CaCl₂, 5 mM KH₂PO₄, 30 mM Hepes-KOH (pH 7.0), 2 mM MgCl₂, 0.25 mM pyridoxal phosphate, 5 mM oxaloacetate, 5 mM DTT, 2 mM NAD, 4 mM ADP, and protoplast lysate in a total volume of 0.8 mL. Reactions were carried out in glass vials fitted with central wells containing 70 μL of 20% (w/v) KOH and closed with self-sealing rubber stoppers. Following 2 min preincubation at 30°C the reactions were started with the injection of 0.2 mL of [1-¹⁴C]glycine (20 mM final concentration, specific activity 0.19 GBq·mol⁻¹) and were stopped after 20 min with the injection of 0.2 mL of 2.5 M H₂SO₄. The vials were left overnight at room temperature to allow absorption of the evolved CO₂ and then the radioactivity in an aliquot of the KOH was determined by liquid scintillation counting.

RESULTS

GDC antibody gave single bands on Western blots of SDS-polyacrylamide gels of crude leaf extracts from pea and wheat. The band corresponded to a protein with a mol wt of approximately 98,000, which concurs with previous reports on the size of the P-protein (results not shown). The antibody was therefore taken to be a reliable probe for the detection of P-protein in both wheat and pea leaves.

Figure 1a is a low power electron micrograph of a section through a pea leaf that has been immunolabeled with the GDC antibody (see “Materials and Methods” for details). There is a significant population of mitochondria in both the mesophyll cells and in the nonphotosynthetic vascular parenchyma cells of the pea leaf. At higher magnification (Fig. 1b, mesophyll cell mitochondria; Fig. 1c, vascular parenchyma cell mitochondria), the immunogold label due to the presence of GDC P-protein can clearly be seen to be localized in the mitochondria in both cell types, although the mesophyll cell mitochondria are more densely labeled.

Sections through a relatively mature region (8 cm from the basal meristem) of a wheat primary leaf show the presence of discrete populations of mitochondria in mesophyll (Fig. 2a) and vascular parenchyma cells (Fig. 2b). The mesophyll cell mitochondria (Fig. 2a) are more densely labeled by GDC antibody than those of the vascular cells (Fig. 2b).

The density of labeling on the mitochondria in all of the cells examined (Table I) was significantly greater than that on the background (P < 0.001 for wheat, P < 0.01 for pea). The highest density of label occurs in the mesophyll cell mitochondria of both species, while the vascular parenchyma (wheat and pea) and epidermal cell (pea) mitochondria are labeled the least (Table I). In pea leaves, two cells of the bundle sheath and two guard cells were examined, and, although the sample was too small for statistical analysis (16 mitochondrial profiles in each sample), the labeling density appears to be intermediate between that of the mesophyll cells and of the nonphotosynthetic vascular parenchyma and epidermal cells (Table I). The higher density of immunogold labeling of wheat, as compared to pea, is of no physiological importance since a smaller size of gold probe was used for wheat and this tends to increase the intensity of labeling.

The activity of GDC (per cell) in wheat mesophyll cells increases with increasing distance from the leaf base (Fig. 3). The density of immunogold labeling of GDC within these mitochondria shows a similar relationship with cell maturity. Both the activity and the intensity of immunogold labeling increase most rapidly in the region beyond 4.0 cm from the leaf base. In contrast, the density of immunolabeling of GDC in vascular parenchyma cells decreases with leaf cell development.

DISCUSSION

We have demonstrated for the first time that the leaves of both monocotyledonous (wheat) and dicotyledonous (pea) C₃ plants contain populations of mitochondria which are heterogeneous with respect to the presence of the P-protein subunit of GDC. Mature mesophyll cells, which possess fully functional chloroplasts capable of photosynthesis and photorespiration, contain mitochondria which are densely labeled for P-protein. Nonphotosynthetic epidermal and vascular parenchyma cells have a much lower density of labeling on their mitochondria. Furthermore, the bundle sheath and guard cells appear to contain a level of P-protein intermediate between these two extremes. These results suggest that a positive relationship may exist between the mitochondrial concentration of GDC and the photosynthetic activity of the cell. To determine whether this is the case, or whether the distribution of GDC is merely a reflection of the differentiation into different cell types we studied the natural developmental gradient of cells that forms along the length of light-grown wheat leaves. All cells of a wheat leaf are derived from divisions within a basal meristem. Cells are continually displaced away from the base and thus increase in their maturity towards the leaf tip (1). It is already well established that the photosynthetic status of the mesophyll cells changes considerably from the immature cells at the leaf base which are unable to fix CO₂ through to mature, fully functional mesophyll cells at the leaf tip (16, 21). The results presented above clearly show that the activity of GDC in the mesophyll cells increases with distance from the leaf base. That is, as the
mesophyll cells mature and develop photosynthetically, the cellular activity of GDC increases. This pattern of increase in GDC activity exactly parallels that of other photorespiratory enzymes, such as chloroplast glutamine synthetase and peroxisomal glycollate oxidase (21) so that the ratio of these three enzymes remains constant throughout cell development. Furthermore, this pattern of development contrasts completely with that of other mitochondrial enzymes, such as cytochrome oxidase, succinate dehydrogenase, and glutamate dehydrogenase. The activities of these enzymes are relatively high in the basal meristematic cells and then increase to reach a constant level at 4.0 cm from the base (21). Thus, control in the cellular level of GDC appears to be independent of that of other mitochondrial enzymes and is coordinated to that of the photorespiratory enzymes.

The density of immunogold labeling of mesophyll cell
GLYCINE DECARBOXYLASE LOCALIZATION IN CELLS OF C₃ LEAVES

Figure 2. Immunogold labeling of a mature wheat leaf section by antibodies to the P-protein of the glycine decarboxylase complex at a dilution of 1:600. a, Mesophyll cell (×150,000). b, Vascular parenchyma cell (×150,000). C = chloroplast, M = mitochondrion. Bars = 0.5 μm.
The concentration becomes nonlinear at high mitochondrial levels of P-protein. Nevertheless, an earlier study of the development of isocitrate lyase activity in glyoxysomes has shown a direct correlation between the density of immunogold labeling of the organelles and the activity of the enzyme (7). We are therefore undertaking a stereological analysis to determine whether there are any developmental changes in the mitochondrial volumes or numbers within the cell. The labeling of GDC in vascular parenchyma mitochondria decreases with distance from the leaf base, i.e. with increasing cell maturity. This decrease may reflect the increased specialization of the cells away from the relatively undifferentiated cells in the basal region of the leaf. There is a distinct difference between the vascular and mesophyll cells in the way that the concentration of GDC changes with cell development. This serves to distinguish between a purely developmental, or temporal, influence on GDC distribution and a more specific control from within the mesophyll cell. The overriding influence appears, therefore, to be that of the photosynthetic status of the host cell. There is clearly a positive relationship between the concentration of GDC in the mitochondria and the photosynthetic development of the cell. This relationship occurs across a range of different cell types and throughout a developmental transition from nonphotosynthetic to mature mesophyll cells.

The P-protein of the GDC complex is responsible for the first step in the sequence of reactions leading to glycine oxidation within the mitochondria (12, 22). In the absence of this subunit, the mitochondria are unable to oxidize glycine (12). The fact that all of the mitochondria examined in this study contained significant amounts of label indicates that they may all be capable of some degree of glycine metabolism, although immunogold labeling detects only the protein and does not prove that it is catalytically active. It is possible that all cells need some GDC activity in order to turn over tetrahydrofolate (4), but the source of the substrate in nonphotosynthetic cells is uncertain. Epidermal and xylem parenchyma cells of *Lycopersicon esculentum* leaves have recently been found to contain ferredoxin-dependent glutamate synthase (2) and chloroplast glutamine synthetase (3), the enzymes necessary for chloroplast ammonia assimilation. This finding, together with our data, indicates that these cells possess at least a limited capacity to release and reaminate ammonia from glycine, providing this substrate is available. The photosynthetic status of guard cells is somewhat controversial. Recent reports have provided evidence of the presence of RuBP carboxylase protein (25), although *14CO2*-labeling patterns indicate limited carboxylation by RuBP carboxylase (11). Furthermore, guard cells appear to have reduced levels of PSI1 components in comparison with mesophyll cells (26). The intermediate level of P-protein found here in the mitochondria of guard and bundle sheath cells indicates that their capacity for metabolism of photosynthetic substrates may be between that of those in mesophyll and epidermal or vascular cells.

The variations in mitochondrial P-protein distribution reported here have particular relevance to studies using isolated leaf mitochondria. The response of isolated pea leaf mitochondria to glycine in the presence of TCA cycle substrates, for example, has prompted the hypothesis that the TCA cycle enzymes have access to some respiratory chains that are inaccessible to GDC (6, 8, 24). An alternative explanation is...
that the leaf mitochondrial preparation contains a mixed population of mitochondria extracted from both photosynthetic and nonphotosynthetic cells which, as we have shown, vary considerably in the level of GDC and hence in their capacity to oxidize glycine. This is particularly so for the pea leaves examined since the tissue was harvested at a similar stage to leaves used to produce isolated mitochondria for oxidative studies. Our developmental studies with wheat leaves (21) (this paper) further suggest that the activity of GDC relative to other mitochondrial enzymes, e.g., cytochrome oxidase, varies with the age of the cell. This creates an additional source of heterogeneity in the mitochondrial preparation in that even fully expanded pea leaves will contain cells of different ages. We are currently examining isolated leaf mitochondria to determine whether the heterogeneity within the leaf is still maintained following extraction and purification of the organelles.

These results indicate that the cells of C3 leaves may be capable of regulating the mitochondrial levels of P-protein according to their photosynthetic capacity. It has been shown that GDC protein constitutes a major proportion of the total matrix protein of isolated pea leaf mitochondria (5). Clearly, a regulatory mechanism that could control the production of GDC protein in cells unable to photorespire would be of economic importance to the plant.

LITERATURE CITED