PLANT $\gamma$-GLUTAMYL HYDROLASES AND FOLATE POLYGLUTAMATES: CHARACTERIZATION, COMPARTMENTATION, AND CO-OCCURRENCE IN VACUOLES

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$\gamma$-Glutamyl hydrolase (GGH, E.C. 3.4.19.9) catalyzes removal of the polyglutamyl tail from folyl and $p$-aminobenzoyl polyglutamates. Plants typically have one or a few GGH genes; *Arabidopsis* has three, tandemly arranged on chromosome 1, which encode proteins with predicted secretory pathway signal peptides. Two representative *Arabidopsis* GGH proteins, AtGGH1 and AtGGH2 (the At1g78660 and At1g78680 gene products, respectively) were expressed in truncated form in *Escherichia coli* and purified. Both enzymes were active as dimers, had low $K_m$ values (0.5 to 2 µM) for folyl and $p$-aminobenzoyl pentaglutamates, and acted as endopeptidases. However, despite 80% sequence identity, they differed in that AtGGH1 cleaved pentaglutamates mainly to di- and triglutamates, whereas AtGGH2 yielded mainly monoglutamates. Analysis of subcellular fractions of pea leaves and red beet roots established that GGH activity is confined to the vacuole and that this activity, if not so sequestered, would deglutamylate all cellular folylpolyglutamates within minutes. Purified pea leaf vacuoles contained an average of 20% of the total cellular folate, compared to ~50% and ~10%, respectively, in mitochondria and chloroplasts. The main vacuolar folate was 5-methyltetrahydrofolate, of which 51% was polyglutamylated. In contrast, the principal mitochondrial and chloroplastic forms were 5-formyl- and 5,10-methenyltetrahydrofolate polyglutamates, respectively. In beet roots, 16 to 60% of the folate was vacuolar, and was again mainly 5-methyltetrahydrofolate, of which 76% was polyglutamylated. These data point to a hitherto unsuspected role for vacuoles in folate storage. Furthermore, the paradoxical co-occurrence of GGH and polyglylutamates in vacuoles implies that the polyglutamates are somehow protected from GGH attack.

In plants, as in other organisms, tetrahydrofolate (THF) and its derivatives – collectively termed folates – usually have $\gamma$-linked polyglutamyl tails of up to about seven residues attached to the first glutamate (Fig. 1A) (1, 2). The tail critically affects the cofactor activity and transport of folates because most folate-dependent enzymes prefer polyglutamates while most carriers prefer monoglutamates (3, 4). By favoring protein binding, the tail also enhances folate stability since bound folate is less prone to oxidative degradation (1). The enzyme that adds the polyglutamyl tails, folylpolyglutamyl synthetase, has been well studied in plants and shown to have mitochondrial, plastidial, and cytosolic isoforms (5, 6). Less is known for plants about the enzyme that removes the tails, $\gamma$-glutamylhydrolase (GGH), although soluble GGH activity (5, 7) and GGH-like mRNAs (8, 9) clearly occur widely.

Biochemical characterization of plant GGHs has so far been limited to crude extracts (9, 10) or a mixture of isoforms (11) and has produced conflicting results on the enzyme’s structure and mode of action. Furthermore, the subcellular location of plant GGH is unclear; cytosolic (11) or extracellular (12) sites have been proposed, but the vacuole is another a priori possibility (11, 13). Wherever it is, plant GGH must be sequestered away from folate polyglutamates, since extractable GGH activities are typically enough to deglutamylate all cellular folate within minutes (7, 10, 11) and treatments that disrupt cellular compartment-
ation cause massive deglutamylation of folates in situ (14).

Nor is much known about the subcellular distribution in plants of the folate polyglutamate substrates of GGH, i.e., about the glutamyl tail lengths, one-carbon (C1) substituents, and oxidation state of the folates in different organelles. The only data are for pea leaves, in which penta- and tetr glutamates of 5-formyl-THF and THF appear to predominate in mitochondria, and 5-methyl-THF (of unknown tail length) elsewhere (15-17). There are, however, some data for pea on the distribution of total folate. Mitochondria, plastids, and a fraction comprised mainly of the cytosol and vacuole accounted for 11-30%, 3-11%, and 60-80%, respectively, of leaf folate (17-19). Besides not distinguishing among folate forms, these data leave open the key question of whether vacuoles contain folates.

Knowing whether folates or GGH occur in vacuoles is important for several reasons. If vacuoles store folates, the transport or storage process might be engineered to enhance folate accumulation (20-22). However, if GGH is vacuolar, the situation could be as in mammals, where lysosomes do not store folates but import folate polyglutamates, hydrolyze them, and export monogluta mates (23, 24). GGH also hydrolyzes the p-aminobenzoate (pABA) polyglutamates formed by oxidative degradation of folates (11, 25), yielding p-aminobenzoylglutamate (pABAGlu1). This may be the first step in the salvage of pABA for folate re-synthesis, a potentially crucial but unexplored process (22).

In this study, we used recombinant Arabidopsis GGHs to clarify the features of this enzyme in plants, and showed that GGH is confined to vacuoles. We also determined the types and tail lengths of folates in organelles. This analysis revealed high levels of 5-methyl-THF polyglutamates in vacuoles.

**EXPERIMENTAL PROCEDURES**

*Chemicals and Reagents—Folic acid (PteGlu), other folates, pABA polyglutamates (pABAGlu), and poly-γ-L-glutamates (γ-Glu) were from Schircks Laboratories (Jona, Switzerland). 5,10-Methenyl-THF polyglutamates were made from the corresponding 5-formyl compounds (26). [3',5',7,9-3H]-Folic acid was from Moravek Biochemicals (Brea, CA). Macerozyme R-10 and Cellulase Onozuka R-10 were from Yakult Honsha (Tokyo, Japan). Ni2+-nitriloacetic acid superflow resin was from Qiagen. Percoll was from Amersham Biosciences. Other biochemicals were from Sigma.

*Plant Material—Pea plants (Pisum sativum cv. Laxton’s Progress 9) were grown in vermiculite at 16-18°C in 12-h days (75 µE m-2 s-1, Cool Light fluorescent tubes) for 12-18 days. Water was added only at sowing. Fresh red beet (Beta vulgaris) roots were purchased locally and used within 2 days.

*Subcellular Fractionation of Pea Leaves—Mitochondria and chloroplasts were purified on Percoll gradients (27, 28). Protoplasts, vacuoles, and a cytosol-enriched fraction were prepared by modifying published methods (29, 30). Briefly, expanded leaves were harvested after 12-24 h in the dark or dim light, cut into 1-mm strips, and washed in 10 mM MES/NaOH, pH 5.5, 0.5 M sorbitol, 5 mM CaCl2, 0.05% (w/v) PVP-40. A previously clarified (20,000 × g, 20 min) digestion medium containing 20 mg ml-1 cellulase and 5 mg ml-1 macerozyme in the same buffer was then added (10 ml per g of tissue) followed by incubation at 25°C for 4 h in white fluorescent light (150 µE m-2 s-1) with rotary shaking (60 rpm). The released protoplasts were filtered through a 70-µm nylon mesh and purified on a three-step sucrose-sorbitol gradient as described (29) except that the buffer contained 5 mM CaCl2 and the density of the bottom layer was increased by adding 10% (v/v) PVP-40. A cytosolic fraction was prepared as described (29) from protoplasts washed in 10 mM MOPS-NaOH, pH 7.0, 0.5 M sorbitol, 5 mM CaCl2. To obtain vacuoles, washed protoplast suspensions (3-4 mg chlorophyll ml-1 corresponding to 4.5 × 10^7 protoplasts ml-1) were: (i) rapidly diluted 1:10 in 100 mM K-phosphate, pH 8.0, 1 mM dithiothreitol, 1 mM MgCl2, 0.05% (w/v) PVP-40; (ii) gently stirred for 4 min; and (iii) loaded in 1.5-ml aliquots onto a three-step gradient consisting of 5% Ficoll (5 ml, bottom), 3% Ficoll (2 ml, middle), and 0.5% Ficoll (1.5 ml, top), each in the above buffer. This procedure was scaled up as necessary. After centrifugation (30 min, 1,000 × g, swinging bucket rotor without braking), intact vacuoles were recovered at the 3%-0.5% Ficoll interface and used without further treatment.
Organelle preparations for folate analysis were supplemented with 10 mM β-mercaptoethanol, frozen in liquid N\textsubscript{2}, and stored at −80°C. Enzymes were extracted from organelles by freezing in liquid N\textsubscript{2} and thawing at 30°C (3-5 cycles), then centrifuging at 16,000 × g for 10 min at 4°C. These extracts, and cytosol fractions, were desalted on PD-10 columns (Amersham Biosciences) equilibrated in 100 mM K-phosphate, pH 6.0, 10% glycerol, 10 mM β-mercaptoethanol, 10 mM ascorbic acid, and stored at −80°C after freezing in liquid N\textsubscript{2}. GGH was assayed as outlined below, and the marker enzymes α-mannosidase, NADP-linked glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fumarase, and methylene tetrahydrofolate reductase (MTHFR) essentially as described (30–32). Fumarase was assayed in a separate set of freshly-prepared extracts not desalted or desalted in 50 mM Tricine-NaOH, pH 8.4.

**Red Beet Root Vacuoles**—Intact vacuoles were isolated essentially as described (33) except that dithiothreitol replaced β-mercaptoethanol and Histodenz\textsuperscript{TM} replaced Metrizamide. Sigma plant protease inhibitor cocktail was added to all media except when vacuoles were assayed for GGH activity. Routinely, 450 g of roots were peeled, cut into 5-mm slices, and loaded into the slicing apparatus, which was set to a slice thickness of 0.1 mm and operated at ~150 rpm. The slices and released cell contents were collected in 1 l of ice-cold 50 mM Tris-HCl, pH 8.0, containing 1 M sorbitol, 5 mM EDTA, 15 mM dithiothreitol, plus or minus 1 ml/l protease inhibitor cocktail, and filtered through two layers of cheesecloth. The solid residue was sliced and filtered again, and the pooled filtrates were centrifuged at 2,000 × g for 20 min at 4°C. The vacuole-enriched pellets were resuspended in 20 ml 15% (w/v) Histodenz\textsuperscript{TM} dissolved in buffer A (1.2 M sorbitol, 1 mM EDTA, 15 mM dithiothreitol, 25 mM Tris-MES, pH 8.0, plus or minus 1 ml/l protease inhibitor cocktail). To prepare density gradients, 5-ml aliquots of the suspension in 15-ml disposable clinical centrifuge tubes were successively overlayed with 5 ml 10% (w/v) Histodenz\textsuperscript{TM} in buffer A and 2 ml of buffer A. Gradients were centrifuged at 650 × g for 10 min at 4°C and vacuoles were collected from the 0/10% Histodenz\textsuperscript{TM} interface. To remove most of the Histodenz\textsuperscript{TM}, the suspension was added to at least 2 volumes of buffer A and centrifuged at 650 × g for 5 min at 4°C. The final vacuole pellet was resuspended in 200-500 µl resuspension medium or 200 µl GGH assay buffer (see below), frozen in liquid N\textsubscript{2}, and held at −80°C until analysis. Betain contents of vacuoles and samples of the roots they came from were estimated spectrophotometrically (ε\textsubscript{550nm} = 62,000 M\textsuperscript{−1} cm\textsuperscript{−1}) (34). Betain was extracted from freeze-dried root samples by grinding in 1 mM EDTA, 50 mM Tris-HCl, pH 7.5, and centrifuging to clarify.

**Folate Analysis**—Folates were extracted and analyzed by HPLC with electrochemical detection as described (21, 35, 36) with the following modifications. Subcellular fraction samples were routinely thawed, made to 10 ml with extraction buffer (50 mM Hepes-50 mM CHES, pH 7.9, 2% (w/v) sodium ascorbate, 10 mM β-mercaptoethanol), split in two, and processed plus or minus treatment with rat plasma conjugase. The folate-binding column was scaled down from 5 to 1 ml, and the volumes of wash and eluting buffers were reduced proportionately. Beet root samples (8 g) were homogenized in 60 ml of extraction buffer; half the extract was conjugase-treated and half not. The HPLC column and mobile phase buffers were as described (21) with a 55-min (for monoglutamates) or 70-min (for polyglutamates) non-linear elution program. Detector response was calibrated using THF, 5-methyl-THF, 5,10-methenyl-THF, 5-formyl-THF, and PteGlu\textsubscript{1} standards. Tri- and pentaglutamate forms of 5-methyl-, 5-formyl-, and 5,10-methenyl-THF were used to identify retention times of polyglutamates.

**cDNAs and Expression in Escherichia coli**—ESTs for genes At1g78660 (GenBank\textsuperscript{TM} CF653065) and At1g78680 (GenBank\textsuperscript{TM} AY096428) were obtained from the Deutsches Ressourcenzentrum für Genomforschung (Berlin, Germany) and the Arabidopsis Biological Resource Center (Columbus, OH), respectively. Based on sequence alignments, constructs lacking ~20 or ~45 N-terminal residues were designed for each protein (see Supplemental Fig. 1). The corresponding cDNAs were amplified using Pfu polymerase and the primers shown in Table I, digested, and cloned between the NdeI and XhoI sites of pET28b (Novagen, Madison, WI). This fused the hexahistidine tag-containing sequence MGSSHHHHHHHHSSGLVPRGSHM to the N-terminus of the proteins. Constructs were made in E. coli DH10B cells, sequence-verified, and introduced into E. coli BL21-CodonPlus\textsuperscript{®}
(DE3)-RIL cells (Stratagene), which were grown at 27°C in LB medium containing 50 µg ml⁻¹ kanamycin and 20 µg ml⁻¹ chloramphenicol until A₆₀₀ reached 0.6. Isopropyl-D-thiogalactopyranos-side was then added (final concentration 1 mM) and incubation was continued for 3 h at 27°C.

**Protein Purification**—Operations were at 0–4°C. Cells from a 50-ml culture were harvested by centrifugation, resuspended in 1 ml of 50 mM K-phosphate, pH 8.0, 1.5 M NaCl, 10 mM β-mercaptoethanol, and broken in a Mini-BeadBeater (Biospec, Bartlesville, OK) using 0.1-mm zirconia/silica beads. After centrifugation (16,000 × g, 15 min), the supernatant was subjected to Ni²⁺ chelate affinity chromatography (0.5-ml column) following the manufacturer’s protocol. Bound proteins were eluted using 250 mM imidazole and desalted on PD-10 columns in 100 mM K-phosphate, pH 6.0, 10% glycerol, 10 mM β-mercaptoethanol. After storage at 4°C or at −80°C after freezing in liquid N₂, the purified enzymes maintained full activity for several weeks and months, respectively. Protein was estimated by Bradford's method (37) using bovine serum albumin as standard.

**Analytical Ultracentrifugation**—Experiments were run using a Beckman XL-I analytical ultracentrifuge and an AN-60-Ti rotor at 25°C. The proteins were dialyzed into 10 mM Na-acetate, pH 5.5, 1 M NaCl, 1 mM dithiothreitol, 1 mM octyl β-glucoside. Buffer viscosity and density and protein partial specific volume (\(\bar{v}\)) were obtained from SEDNTERP software (http://www.jphilo.mailway.com). The \(\bar{v}\) values for AtGGH1 and AtGGH2 were respectively calculated from amino acid content to be 0.7324 and 0.7351 ml/g at 20°C. In sedimentation velocity studies, sample volume was 0.42 ml, and the reference volume of dialysis buffer was 0.44 ml. The samples were run at 50,000 rpm. Absorption measurements were made at 280 nm for AtGGH1 (0.8 mg/ml) and 230 nm for AtGGH2 (0.1 mg/ml). A single sample was run in each experiment with zero time between scans, \(R_{min}\) was set at 6.0, and the samples were scanned from the earliest time until the boundaries reached the cell bottom. The samples were at thermal equilibrium before starting to spin the rotor, which was accelerated directly to 50,000 rpm. The data were analyzed using the c(s) and c(M) methods found in Sedfit (38) (http://www.analyticalultracentrifugation.com). The sedimentation coefficients calculated from the Sedfit program were converted to S_{20,w} values using Sedfit.

**GGH Assay**—Standard assay mixtures contained folate pentaglutamate (PteGlu₅) or pABAGlu₅ (0.2 mM unless otherwise indicated), 100 mM K-phosphate, pH 6.0, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, and enzyme in a final volume of 100 µl. After incubation for 0-6 h, the reaction was stopped by boiling for 3 min, then centrifuged at 16,000 × g for 10 min. Incubation was at 37°C; no substantial difference in reaction rate was observed in the range 30–42°C. Folate- or pABA-containing analytes in the supernatant were separated by HPLC using a C18 Symmetry column (Waters, 7.5 × 0.46 cm; 3 µm particle size) and quantified by absorption at 282 nm (292 nm for 5-methyl-THF) or by fluorescence (270 nm excitation, 350 nm emission), respectively. The column was eluted at 1.5 ml min⁻¹ with a 10-min linear gradient from 15% to 45% (v/v) methanol in 50 mM Na-phosphate buffer, pH 6.0, containing 8 mM tetra-butylammonium bisulfate (buffer B). γ-Glu₅ and glutamate were derivatized with o-phthalaldehyde (39), separated using the column above connected in series to a C18 Spherisorb ODS2 column (Waters, 15 × 0.46 cm; 5 µm particle size) and isocratic elution with 40% methanol buffer B, and quantified fluorometrically (365 nm excitation, 420 nm emission). For kinetic studies, initial rates of substrate disappearance were measured (before an average of 25% of PteGlu₅ or 3% of pABAGlu₅ had been used). Rates were proportional to enzyme concentration and time. Kinetic constants were calculated from Hanes-Woolf plots. To determine specificity for the various γ-glutamyl bonds in PteGlu₅ or pABAGlu₅, residual substrate and the various PteGluₙ or pABAGluₙ products were determined, and the extent of hydrolysis (\(E\)) was plotted versus the relative concentration (\(R\)) of each PteGluₙ or pABAGluₙ species (40). These terms are defined as follows:

\[
E = \frac{(X\text{Glu}_1 + X\text{Glu}_3 + X\text{Glu}_5 + X\text{Glu}_7)}{(X\text{Glu}_3 + X\text{Glu}_4 + X\text{Glu}_5 + X\text{Glu}_7 + X\text{Glu}_1)}
\]

\[
R = \frac{(X\text{Glu}_n)}{(X\text{Glu}_3 + X\text{Glu}_4 + X\text{Glu}_5 + X\text{Glu}_7 + X\text{Glu}_1)}
\]

where \(X\text{Glu}_n\) = PteGluₙ or pABAGluₙ. The slope of the line corresponding to each product measures...
the relative extent of its formation, and so indicates the \( \gamma \)-glutamyl bond specificity of the enzyme.

**RESULTS**

**GGH-like Genes in Arabidopsis and Other Plants**—BLAST searches of genome and EST databases at GenBank™ and TIGR indicated that higher plants have one or a few genes encoding proteins similar to mammalian GGHs. For instance, rice and maize appear to have one gene, soybean and cotton two, \textit{Arabidopsis} three, and tomato four. \textit{Arabidopsis} is thus typical in having a small GGH gene family.

The three \textit{Arabidopsis} genes are tandemly arranged on chromosome 1, and specify proteins (AtGGH1, 2, and 3) that are \(-35\%\) identical to human GGH and \(70-80\%\) identical to each other. As shown in Supplemental Fig. 1, the \textit{Arabidopsis} proteins share many features with mammalian GGHs, including a predicted secretory pathway signal peptide (41, 42), at least one \( N \)-glycosylation motif (13), and the conserved cysteine and histidine residues that are catalytically essential in human GGH as well as six other conserved residues that may contribute to catalysis or substrate binding (43, 44). In contrast to these similarities, the AtGGH3 sequence has two short insertions near the N-terminus that have no clear-cut counterparts in mammalian GGHs and are absent from all other available plant GGH sequences. Since AtGGH3 appears to be unique to \textit{Arabidopsis} and thus not of general interest it was not further studied. This enzyme is also far less strongly expressed than AtGGH1 or 2 (Orsomando and Hanson, unpublished).

**Kinetic Characterization of GGH Proteins**—Recombinant AtGGH1 and AtGGH2 were expressed in \textit{E. coli}, removing the signal peptide region and replacing it with a sequence containing a histidine tag as was done for human GGH (43). This strategy gave high levels of both proteins, which were purified by Ni\(^{2+}\) affinity chromatography to \(>90\%\) homogeneity as judged by SDS-PAGE (see Supplemental Fig. 2). Two versions of each enzyme were made, differing in how many residues were removed (see Supplemental Fig. 1). As results for both versions were similar we report data only for the shorter one. Enzyme assays were made at pH 6.0, which was shown to be near the optimum for both enzymes (6.5-7.0 for AtGGH1 and 6.5 for AtGGH2) and is also close to the pH of the vacuole (in which GGHs are located, see below).

The mode of action and specificity of purified AtGGH1 and AtGGH2 were studied using the pentaglutamates of folic acid (PteGlu\(_5\)) and \(\gamma\)-ABA (\(\gamma\)ABA\(\gamma\)Glu\(_3\)) as substrates (Fig. 1, B and C). In the initial stage of PteGlu\(_i\) hydrolysis, AtGGH1 formed PteGlu\(_2\) and PteGlu\(_3\) at similar rates, whereas AtGGH2 formed PteGlu\(_1\) and a small amount of PteGlu\(_2\) (Fig. 1B). This indicates that both enzymes have an endopeptidase action, with AtGGH1 showing an almost equal preference for the second and third \(\gamma\)-glutamyl bonds (counting from the folate moiety) and AtGGH2 preferring the first. This difference in specificity was also evident when \(\gamma\)ABA\(\gamma\)Glu\(_3\) was the substrate, with an additional cleavage at lower frequency of the fourth \(\gamma\)-glutamyl bond catalyzed by AtGGH2 (Fig. 1C).

The kinetic constants for AtGGH1 and AtGGH2 with PteGlu\(_i\) or \(\gamma\)ABA\(\gamma\)Glu\(_3\) as substrate are shown in Table II. The \(K_m\) values for PteGlu\(_5\) for both enzymes were lower than those reported for a purified pea GGH preparation, and similar to those found for mammalian GGHs, which are typically \(<2\ \mu\text{M}\). As judged from the \(k_{cat}/K_m\) ratios, both enzymes hydrolyzed \(\gamma\)ABA\(\gamma\)Glu\(_5\) less efficiently than PteGlu\(_5\).

Further distinctions between the two enzymes appeared when long incubation periods were used to define the end-products of the reaction (Fig. 2). When AtGGH1 acted on PteGlu\(_5\), the PteGlu\(_3\) formed initially – but not the PteGlu\(_2\) – was further hydrolyzed to PteGlu\(_1\), so that the final folate products included PteGlu\(_1\) as well as PteGlu\(_2\) and PteGlu\(_3\) (Fig. 2A). The other products were \(\gamma\)-Glu\(_3\), \(\gamma\)-Glu\(_2\), and \(\gamma\)-Glu\(_1\), which were not attacked (Fig. 2B). In contrast, AtGGH2 gave only PteGlu\(_1\) as the final folate product, although some PteGlu\(_2\) accumulated transiently (Fig. 2A). The other products of AtGGH2 cleavage were \(\gamma\)-Glu\(_3\), \(\gamma\)-Glu\(_2\) and glutamate, with \(\gamma\)-Glu\(_2\) and \(\gamma\)-Glu\(_3\) disappearing as the reaction progressed (Fig. 2B). The unexpected formation of \(\gamma\)-Glu\(_3\) is considered below. The results with \(\gamma\)ABA\(\gamma\)Glu\(_3\) as substrate resembled those with PteGlu\(_3\) except that AtGGH1 did not further hydrolyze \(\gamma\)ABA\(\gamma\)Glu\(_3\), and AtGGH2 produced a small, transient peak of \(\gamma\)ABA\(\gamma\)Glu\(_4\) and did not further hydrolyze \(\gamma\)ABA\(\gamma\)Glu\(_2\) (Fig. 2, C and D). The kinetics of appearance of glutamate and \(\gamma\)-Glu\(_3\) (Fig. 2, B and D) indicated that AtGGH1 does not attack \(\gamma\)-glutamyl pept-
ides released from PteGlu or pABAGlu₅, while AtGGH2 does so via exopeptidase action, the early accumulation of γ-Glu₄ being followed by γ-Glu₃ and later by γ-Glu₂ and γ-Glu₁. This was confirmed by using γ-Glu₃ as substrate (not shown).

Formation of γ-Glu₅ by AtGGH2 (Fig. 2, B and D) was not due to cleavage of the bond between glutamate and pABA because there was no decline in total PteGlu₅ or pABAGlu₅ during the reaction (Fig. 2, A and C) and no pteroic acid was released from PteGlu₅ or pABA from pABAGlu₅. This was confirmed by demonstrating that, like mammalian GGHs (13), neither plant enzyme attacks PteGlu₁ or pABAGlu₁ (not shown). The γ-Glu₅ thus presumably came from transpeptidation between the γ-Glu₄ products released from Pte- or pABAGlu₄. Transpeptidase activity is common among peptidases, although not previously reported for GGH. Transpeptidase can also explain why no glutamate counterpart to the pABAGlu₄ formed was seen for AtGGH2 acting on pABAGlu₁ (Fig. 2, C and D).

Native Molecular Mass—Purified recombinant enzymes were analyzed by analytical ultracentrifugation in sedimentation velocity experiments. Each protein yielded a single component in the analysis. For AtGGH1 the S₂₀,ₘ value from the c(s) method was 4.57 S corresponding to a molecular mass of 69.8 kDa calculated using the c(M) method. For AtGGH2 the S₂₀,ₘ was 4.43 S and the calculated mass was 66.0 kDa. These values are close to twice the calculated masses of the recombinant AtGGH1 and AtGGH2 polypeptides (36.7 and 36.4 kDa, respectively), indicating that both enzymes exist as dimers, as does human GGH (44). In this connection, it is noteworthy that the dimer interface regions of the human enzyme (44) are substantially conserved in the plant sequences (see Supplemental Fig. 1).

Subcellular Localization of GGH Activity—GGH was localized by cell fractionation and enzyme assay. Pea leaves were used because they are the tissue of choice for obtaining high yields of intact vacuoles and other organelles (27-29) and have been the object of much prior work in folate biochemistry (11, 15-19). The distribution of marker enzyme activities confirmed that purified chloroplasts, mitochondria, and vacuoles were essentially uncontaminated by other fractions (Fig. 3). The vacuole fraction contained ≤1-2% of the total activities of the cytosol marker MTHFR and the peroxisomal marker catalase (not shown). (Such slight contamination of vacuole preparations is too small to complicate interpretation of the vacuolar folate data to be presented below.) The distribution of GGH activity closely paralleled that of the vacuolar marker α-mannosidase (Fig. 3), indicating that GGH is an exclusively vacuolar enzyme. To corroborate this result, we analyzed vacuoles from red beet roots, a classical plant vacuole system. The ratios between GGH activity and the solely vacuolar pigment betanin (34) in vacuoles and whole roots were, respectively, 29.8 ± 3.5 and 21.0 ± 3.3 pmol min⁻¹ μmol⁻¹ (mean ± S.E.), indicating that GGH activity is entirely vacuolar. GGH activities in beet vacuoles were far lower than those in pea vacuoles (11 ± 1 versus 2,320 ± 420 pmol min⁻¹ mg⁻¹ protein, respectively).

Subcellular Localization of Pea Leaf Folates—Purified organelle preparations and mesophyll protoplasts were first analyzed after removing polyglutamyl tails to enable identification and quantification of the types of folate (i.e., THF and its C₁₂-substituted forms). The protoplast values (Fig. 4, top frame) agree with published total folate contents of pea leaves (16-19), and show a typical distribution of folate types for leaves of pea and other plants (5, 16). (The acidic HPLC mobile phase used in the analysis converts 10-formyl-THF to 5,10-methenyl-THF, so that the 5,10-methenyl-THF measurements include 10-formyl-THF plus any preexisting 5,10-methenyl-THF.) The total folate levels found in mitochondria and chloroplasts (about 50% and 10%, respectively, of cellular folates) also agree with literature values (16-19), as does the finding that 5-formyl-THF is the major folate in mitochondria (16, 17). The most striking result is that vacuoles contain a substantial amount of folate, almost all as 5-methyl-THF (Fig. 4, bottom frame). That this is not contamination by other fractions is attested by the enzyme marker data (see above) and by the distribution of folate types, which differs from those of other fractions (Fig. 4). The absence of significant contamination was further confirmed by adding tracer [³H]folic acid to the protoplast lysates from which vacuoles were isolated; the purified vacuoles accounted for ≤0.5% of the ³H added (data not shown).

To estimate the percentage of total cellular folate and 5-methyl-THF present in vacuoles, the folate contents of vacuole preparations and the...
protoplasts from which they came were expressed relative to the activity of α-mannosidase, which is specific to vacuoles in plants (45). Among three separate preparations, an average of 20% of total folate was vacuolar, and 38% of the 5-methyl-THF (Table III).

Polyglutamylation of Pea Leaf Organellar Folates—The extent of polyglutamylation was determined for the major folate in each organelle (Fig. 5). The main mitochondrial folate, 5-formyl-THF, existed mainly as penta- and hexaglutamates (similar to a previous report, Ref. 15) as did 5,10-methenyl-THF in chloroplasts. Half the vacuolar 5-methyl-THF was also polyglutamylated although the average tail length was shorter than in the other organelles. The survival of any polyglutamylation folate in the vacuole was unexpected as GGH activity is in theory high enough to deglutamylate most of the vacuolar folate in less than a minute (see “Discussion” for the calculation).

To confirm that the persistence of vacuolar 5-methyl-THF polyglutamates is not simply because plant GGHs do not attack them, we compared 5-methyl-THF pentaglutamates and PteGlu5 as substrates for purified AtGGH1 and 2, and for the GGH activity in pea vacuole extracts. At the physiological concentration of 1 µM, both substrates were hydrolyzed at similar rates by all three enzyme preparations. The observed activities (nmol min⁻¹ mg⁻¹ protein, with 5-methyl-THF pentaglutamate and PteGlu₅, respectively) were: AtGGH1 3,690 and 5,200; AtGGH2 3,590 and 4,120; pea GGH 1.9 and 2.8.

Analysis of Beet Root Folates—To explore the generality of the findings above, we analyzed red beet roots and their vacuoles. The principal folate in root tissue was 5-methyl-THF, in accord with published data for beets and other storage organs (46) (Fig. 6A). As with pea vacuoles, beet vacuoles contained substantial levels of 5-methyl-THF (Fig. 6A). The amounts of 5-methyl-THF in beet vacuoles were quantified by relating the folate contents of vacuole preparations and root tissue to their betanin contents (Table IV). In five independent preparations, from 16 to 60% of the 5-methyl-THF was vacuolar. The vacuolar 5-methyl-THF was 76% polyglutamylated, with di- and pentaglutamates predominant (Fig. 6B). Since beet vacuoles also contain GGH, the situation in beet is like that in pea: folyl polyglutamates and the enzyme that hydrolyzes them co-exist in vacuoles.

DISCUSSION

Our results establish that plant GGHs are broadly similar in structure and catalytic properties to those of mammals, as suggested by other reports (8-11). Like mammalian GGHs (13, 47), the Arabidopsis enzymes studied are dimers of ~300-residue polypeptides, have mildly acidic pH optima, attack both pABA and folate polyglutamates, and show different cleavage patterns despite having similar amino acid sequences. AtGGH1 and AtGGH2 both act as endopeptidases but they prefer different bonds and only AtGGH2 attacks PteGlu5. Since PteGlu5 is a major product of AtGGH1 action, the two enzymes, in a sense, complement each other. Such complementarity may have confounded specificity studies of plant GGHs made with crude extracts or mixed isoforms (8, 9). AtGGH2 also shows exopeptidase and transpeptidase action on γ-Glu₅ products.

The Arabidopsis GGH proteins and all other plant GGH sequences available have an N-glycosylation motif in a conserved position, suggesting that they exist naturally as glycoproteins, as do mammalian GGHs (13). Even though mammalian GGHs have four or more potential N-glycosylation sites and are heavily glycosylated, the recombinant proteins produced in E. coli have similar biochemical characteristics to their glycosylated counterparts (48-50). It is therefore likely that the E. coli-derived Arabidopsis GGHs used in our study faithfully mirror the properties of the enzymes as they exist in planta.

Our finding that pea GGH activity is vacuolar is supported by the recent detection of AtGGH2 and AtGGH3 proteins in Arabidopsis leaf vacuoles (42) and fits with the presence of signal peptides, which occur in many vacuole-associated proteins (42, 51) and participate in vacuolar sorting (52). The vacuolar site parallels the lysosomal location of intracellular mammalian GGH (13, 47), for lysosomes and central vacuoles of plants are both lytic compartments (53). An exclusively vacuolar location seems hard to reconcile with previous reports that GGH is cytosolic or extracellular, but this is not the case. The cytosolic location was inferred by fractionating extracts prepared by chopping, which breaks many vacuoles, and in fact 19% of the GGH activity was in the vacuole fraction (11). The apoplastic location was based on there being a GGH-like protein in extracellular wash
fluid of soybean leaves; GGH activity was not assayed (12). Isolation of the corresponding cDNA (8) and cognate ESTs has since shown that this protein, unlike all other plant GGH sequences, lacks two catalytically essential residues (Cys110 and His220 in the human enzyme) (43). It thus appears that soybean cells secrete into the apoplast an unusual GGH-like protein, not an active GGH enzyme.

Our analyses of organellar folates show that each organelle has a distinct folate profile. The mitochondrial profile is dominated by 5-formyl-THF, which is not a C1 donor but an inhibitor of folate-dependent enzymes (54). 5-Formyl-THF is recycled to the active C1 folate pool by a cyclo-ligase that is mitochondrial in plants (55). The chloroplast folate pool is richest in 5,10-methenyl-THF (which is generated during our analysis from 10-formyl-THF) but also contains 5-methyl-THF, whose occurrence in chloroplasts has previously been inferred from the presence of the enzyme that uses it, methionine synthase (56). The folate pools of both leaf and storage root vacuoles proved to consist almost entirely of 5-methyl-THF, suggesting a storage role for this folate. 5-Methyl-THF seems a plausible candidate for storage because it is quite stable and is readily converted to other C1 folates via the reversible MTHFR reaction in plants (32). Although vacuolar storage pools of folate have not been reported before, their existence might have been predicted from the lack of correlation between folate levels and C1 metabolic activity, and the wide variation in folate levels between comparable tissues from different species (57). Beet roots are a good example – although metabolically quiescent their folate levels are as high as those in green leaves, and far higher than those of other storage roots (57).

The observed polyglutamylation of vacuolar folates is paradoxical because the glutamyl tail is not expected to survive long in the presence of the GGH activity, as the following calculation shows. For pea leaves, assuming that the vacuole is 70% of the water volume (58), and that total folate content is 5 nmol g⁻¹ fresh weight (17), the folylpolyglutamate level (Table III and Fig. 5) would be ~1 nmol ml⁻¹. The vacuolar GGH activity (2.3 nmol min⁻¹ mg⁻¹ protein, see Fig. 3) is equivalent to ~8 nmol min⁻¹ ml⁻¹ vacuolar contents at Vmax or ~4 nmol min⁻¹ ml⁻¹ at a folate concentration of ~1 nmol ml⁻¹ (estimated from the vacuolar folate concentration above, the folate and GGH contents expressed per unit protein, and the Km values for the Arabidopsis enzymes). Under steady-state conditions, the vacuolar folyl polyglutamates therefore have a predicted half-life of ~7 sec. A similar calculation for beet root vacuoles indicates a polyglutamate half-life of ~5 min. These calculations assume that pea and beet GGHs behave similarly to a mixture of the Arabidopsis enzymes. Partial characterization of the activities in pea and beet vacuole extracts supported this assumption. The calculations also assume that GGH and folyl polyglutamates show no tendency to be segregated into distinct vacuole subpopulations within cells.

Chloroplasts and mitochondria contain isoforms of folylpolyglutamate synthetase (6) as well as the ATP that this enzyme requires. The presence of polyglutamates in these organelles is therefore easily explained by in-situ synthesis from monoglutamyl folates. Not so for vacuoles, which most probably contain neither the synthetase (6) nor ATP (59). For vacuoles it is thus necessary to invoke – as for mammalian lysosomes (23, 24) – import of folylpolyglutamates by a carrier-mediated process.

The co-occurrence of folylpolyglutamates and GGH in vacuoles could be explained by the presence of a potent GGH inhibitor, or by folate-binding proteins that protect polyglutamates from hydrolysis. There is so far no experimental evidence for or against either possibility. However, inhibiting GGH action on folylpolyglutamates would also stop pABA polyglutamate hydrolysis and so could disrupt folate recycling. Folate-binding proteins are therefore perhaps a more attractive solution. Various such proteins have been characterized in mammals (60) and shown to protect bound folates against both cleavage of the polyglutamyl tail and oxidative degradation (1, 39).

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REFERENCES


**FOOTNOTES**

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1The abbreviations used are: pABA, *p*-aminobenzoic acid; pABAGlu, *p*-aminobenzoylmonoglutamate; pABAGluₙ, *p*-aminobenzoypolyglutamate; C₁, one-carbon; GAPDH, NADP-linked glyceraldehyde-3-phosphate dehydrogenase; GGH, γ-glutamyl hydrolase; γ-Gluₙ, poly-γ-L-glutamate; MTHFR, methylene-tetrahydrofolate reductase; PteGlu₁, folic acid; PteGluₙ, folate polyglutamate; THF, tetrahydrofolate.
FIGURE LEGENDS

FIG. 1. Structures of GGH substrates and the bond cleavage specificity of *Arabidopsis* GGHs. *A*, the structure of folyl and *p*ABA polyglutamates (the folate shown is THF). One-carbon units at various oxidation levels (formyl, methyl, etc.) are attached to *N*5 and/or *N*10 of the folate moiety. Oxidative degradation of folyl polyglutamates results in cleavage of the C9-*N*10 bond, yielding a pterin and *p*-amino-benzoylglutamate (*p*ABAGlu1) with a polyglutamyl tail. *B* and *C*, progress curves for the initial stages of hydrolysis of PteGlu5 (*B*) and *p*ABAGlu5 (*C*) by purified recombinant AtGGH1 or AtGGH2. The substrate concentration supplied was 0.2 mM. Data are presented as plots of relative concentration of each reaction product versus extent of reaction (see “Experimental Procedures”) and are pooled values from two representative experiments.

FIG. 2. Kinetics of product formation during the prolonged action of AtGGH1 or AtGGH2 on pentaglutamate substrates. Initial substrate concentrations were 0.2 mM. Data are presented in units of nmol/20-µl reaction. *A*, folate-containing products formed from PteGlu5. *B*, glutamate and γ-Glu5 products formed from PteGlu5. *C*, *p*ABA-containing products formed from *p*ABAGlu5. *D*, glutamate and γ-Glu5 products formed from *p*ABAGlu5. Numerals (1 to 5) by curves indicate the number of glutamyl residues in each product. Data are from representative individual experiments, which were repeated at least three times with similar results.

FIG. 3. Localization of GGH in pea leaf vacuoles by subcellular fractionation. Chloroplasts (CP), mitochondria (M), and vacuoles (V) were purified by density gradient centrifugation. A fraction enriched in cytosol and vacuole contents (CS+V) was prepared from pea leaf protoplasts by pelleting intact organelles. The specific activities (units mg⁻¹ protein) of GGH (measured using *p*ABAGlu5) and marker enzymes were assayed in each fraction. Markers were: α-mannosidase (vacuole); NADP-linked glyceraldehyde-3-phosphate dehydrogenase (GAPDH, chloroplast); fumarase (mitochondrion); and methylene-tetrahydrofolate reductase (MTHFR, cytosol). The asterisk indicates that a trace of MTHFR activity was detectable in vacuoles, but was too low to quantify; it represented ≤2% of the total activity of the protoplasts. Data are means and S.E. of data from three to ten independent preparations of each fraction.

FIG. 4. Types of folates in organelles from pea leaves. Mitochondria, chloroplasts, and vacuoles were purified by density gradient centrifugation and shown to be free of significant contamination by other fractions, as in Fig. 3. Mesophyll protoplasts were analyzed for comparison. Folates were deglutamylated before HPLC analysis with electrochemical detection. Data are means and S.E. values from three independent organelle or protoplast preparations. In addition to the folates shown, small amounts (~2% of total folate) of folic acid and 10-formyldihydrofolate were found in chloroplasts and mitochondria, respectively. 5-CH₃-THF; 5-methyl-THF; 5-CHO-THF; 5-formyl-THF; 5,10=CH-THF, 5,10-methenyl-THF.

FIG. 5. Distribution of polyglutamyl tail lengths of the main folate types in organelles from pea leaves. The organelle preparations analyzed were the same as in Fig. 4. HPLC analysis with electrochemical detection was used to analyze the tail length distribution for the predominant type of folate in each organelle, i.e., 5-formyl-THF (5-CHO-THF) in mitochondria, 5,10-methenyl-THF (5,10=CH-THF) in chloroplasts, and 5-methyl-THF (5-CH₃-THF) in vacuoles. Data are means and S.D. of data from three independent organelle preparations.

FIG. 6. Analysis of folate types and polyglutamyl tail lengths in red beet roots and vacuoles. Vacuoles were purified by density gradient centrifugation. Folate types (*A*) and the tail length of 5-methyl-THF (*B*) were analyzed by HPLC with electrochemical detection. Data are means and S.E. (*A*) or S.D. (*B*) for four independent vacuole preparations and samples of the roots from which each preparation was made. 5-CH₃-THF; 5-methyl-THF; 5-CHO-THF; 5-formyl-THF; 5,10=CH-THF, 5,10-methenyl-THF.
### TABLE I

*PCR primers used to clone truncated Arabidopsis GGH sequences into pET28b*

NdeI and XhoI sites are underlined. FL, forward primer for long version; FS, forward primer for short version; R, reverse primer.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtGGH1</td>
<td>FL: GATCGATCCCATATGGAGGCTTCTGAGTGGTTT</td>
</tr>
<tr>
<td>(At1g78660)</td>
<td>GAGGCTTCTGAGTGCTCCTGCGCCGGAT</td>
</tr>
<tr>
<td></td>
<td>FS: GATCGATCCCATATGGTTTGGCTCCTGCGCCGGAT</td>
</tr>
<tr>
<td></td>
<td>R: TGATCCTCGAGTTAGAAACGGGATCTTTGTT</td>
</tr>
<tr>
<td>AtGGH2</td>
<td>FL: GATCGATCCCATATGGCCAAAGGCTGCGACG</td>
</tr>
<tr>
<td>(At1g78680)</td>
<td>GCCAAGGCTGCGACG</td>
</tr>
<tr>
<td></td>
<td>FS: GATCGATCCCATATGGTTATGCTCTGCTCCGGAT</td>
</tr>
<tr>
<td></td>
<td>R: TGATCCTCGAGTTAGAAACGGGATCGTTT</td>
</tr>
</tbody>
</table>

### TABLE II

*Kinetic constants of AtGGH1 and AtGGH2 with PteGlu₅ or pABAGlu₅ as substrate*

Values were determined at 37°C in 100 mM K-phosphate buffer, pH 6.0, 10% (v/v) glycerol, 10 mM β-mercaptoethanol, using substrate concentrations of 0.5-10 μM. Data are means of three independent determinations ± S.E.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>PteGlu₅</th>
<th>pABAGlu₅</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$K_{cat}$</td>
</tr>
<tr>
<td></td>
<td>μM</td>
<td>$s^{-1}$</td>
</tr>
<tr>
<td>AtGGH1</td>
<td>0.79 ± 0.04</td>
<td>19.46 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>1.50 ± 0.22</td>
<td>8.29 ± 0.74</td>
</tr>
<tr>
<td>AtGGH2</td>
<td>0.52 ± 0.07</td>
<td>8.74 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>1.86 ± 0.20</td>
<td>5.51 ± 0.61</td>
</tr>
</tbody>
</table>
**TABLE III**

*Total folate and 5-methyl-THF contents of pea leaf mesophyll protoplasts and vacuoles*

Total folate and 5-methyl-THF were determined in three independent protoplast preparations and in the vacuoles derived from them, and expressed relative to the activities of the vacuolar marker α-mannosidase. One α-mannosidase unit (U) = 1 µmol of substrate hydrolyzed min⁻¹.

| Preparation | Total folate |  | 5-Methyl-THF |  |
|-------------|--------------|------------------|------------------|
|             | Protoplasts  | Vacuoles | Vacuolar folate | Protoplasts  | Vacuoles | Vacuolar 5-methyl-THF |
|             | pmol/U       | %       | pmol/U         | % |
| 1           | 19.4         | 2.4     | 12             | 10.6        | 2.4     | 20 |
| 2           | 20.1         | 5.5     | 27             | 9.6         | 5.0     | 52 |
| 3           | 15.6         | 3.6     | 23             | 7.5         | 3.2     | 42 |

**TABLE IV**

*5-Methyl-THF contents of red beet root tissue and vacuoles*

5-Methyl-THF was determined in independent root tissue samples and in vacuoles derived from them, and expressed relative to the level of the vacuolar marker betanin.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>5-Methyl-THF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Root tissue</td>
</tr>
<tr>
<td></td>
<td>nmol µmol⁻¹ betanin</td>
</tr>
<tr>
<td>1</td>
<td>3.80</td>
</tr>
<tr>
<td>2</td>
<td>4.31</td>
</tr>
<tr>
<td>3</td>
<td>2.16</td>
</tr>
<tr>
<td>4</td>
<td>1.43</td>
</tr>
<tr>
<td>5a</td>
<td>2.08</td>
</tr>
</tbody>
</table>

*Three vacuole preparations were pooled for this analysis.*
Alignment of the deduced protein sequences of the three *Arabidopsis* GGH homologs with that of human GGH (HsGGH, GenBank™ NP003869). AtGGH1, 2, and 3 are encoded by genes At1g78660, At1g78680, and At1g78670, respectively. Identical residues are shaded in **black**, similar residues in **gray**. **Dashes** are gaps introduced to maximize alignment. For the plant sequences, the predicted signal peptide region is **overlined** and potential N-glycosylation motifs (NXS/TX, where X is any residue except Pro) are in **red**. **Blue arrowheads** designate conserved residues that are catalytically essential in human GGH, and **black arrowheads** denote other conserved active site residues that may participate in substrate binding. **Dashed underlines** show the three main regions that form the dimer interface in human GGH. **Asterisks** mark the residue of the recombinant proteins to which the His-tag region of the expression vector was fused.
Steps in purification of recombinant AtGGH1 and AtGGH2 proteins. Histidine-tagged AtGGH1 and AtGGH2 were isolated by Ni$^{2+}$ affinity chromatography under native conditions, and separated by SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie Blue. For each protein, lane 1 was loaded with 10 µg of crude *E. coli* extract protein, lane 2 with 10 µg of the proteins not bound to the resin, and lane 3 with 5 µg of purified protein. The positions of molecular mass standards (kDa) are indicated.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Plant γ-glutamyl hydrolases and folate polyglutamates: Characterization, compartmentation, and co-occurrence in vacuoles
Giuseppe Orsomando, Rocio Diaz de la Garza, Brian J. Green, Mingsheng Peng, Philip A. Rea, Thomas J. Ryan, Jesse F. Gregory III and Andrew D. Hanson

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