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Characterization of the folate salvage enzyme *p*-aminobenzoylglutamate hydrolase in plants

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Abstract

Folates break down *in vivo* to give pterin and *p*-aminobenzoylglutamate (*p*ABAGlu) fragments, the latter usually having a polyglutamyl tail. Pilot studies have shown that plants can hydrolyze *p*ABAGlu and its polyglutamates to *p*-aminobenzoate, a folate biosynthesis precursor. The enzymatic basis of this hydrolysis was further investigated. *p*ABAGlu hydrolase activity was found in all species and organs tested; activity levels implied that the proteins responsible are very rare. The activity was located in cytosol/vacuole and mitochondrial fractions of pea (*Pisum sativum* L.) leaves, and column chromatography of the activity from *Arabidopsis* tissues indicated at least three peaks. A major activity peak from *Arabidopsis* roots was purified 86-fold by a three-column procedure; activity loss during purification exceeded 95%. Size exclusion chromatography gave a molecular mass of ~200 kDa. Partially purified preparations showed a pH optimum near 7.5, a K_m value for *p*ABAGlu of 370 μM, and activity against folic acid. Activity was relatively insensitive to thiol and serine reagents, but was strongly inhibited by 8-hydroxyquinoline-5-sulfonic acid and stimulated by Mn^{2+}, pointing to a metalloenzyme. The *Arabidopsis* genome was searched for proteins similar to *Pseudomonas* carboxypeptidase G, which contains zinc and is the only enzyme yet confirmed to attack *p*ABAGlu. The sole significant matches were auxin conjugate hydrolase family members and the At4g17830 protein. None was found to have significant *p*ABAGlu hydrolase activity, suggesting that this activity resides in hitherto unrecognized enzymes. The finding that *Arabidopsis* has folate-hydrolyzing activity points to an enzymatic component of folate degradation in plants.

Keywords: *Arabidopsis thaliana*; Crucifereae; Folate salvage; Enzyme isolation and characterization; *p*-Aminobenzoylglutamate hydrolase; Folate hydrolase

1. Introduction

Tetrahydrofolate (THF) and its one-carbon derivatives (collectively termed folates) are essential cofactors for one-carbon transfer reactions. Folate molecules consist of pterin **4**, *p*-aminobenzoate (*p*ABA **7**), and glutamate **6** moieties, usually with a short, γ-linked polyglutamyl tail attached to the first glutamate (Fig. 1a). Plants, fungi, and bacteria can synthesize folates but higher animals cannot and so need a dietary supply (Cossins, 2000; Scott et al., 2000).

Folates readily undergo spontaneous oxidative degradation in physiological conditions, yielding a pterin **4** and *p*-aminobenzoylglutamate **5** (*p*ABAGlu) or its polyglutamates (Fig. 1a) (Suh et al., 2001). There is evidence that this breakdown process is particularly active in plants and that, *in vivo*, plants can hydrolyze the resulting *p*ABAGlu **5** moieties to *p*ABA **7** and glutamate **6** (Fig. 1b) (Orsomando et al., 2006). Acting in concert with a reductive reaction that recycles the pterin fragment to a folate synthesis precursor, *p*ABAGlu **5** hydrolysis enables complete salvage

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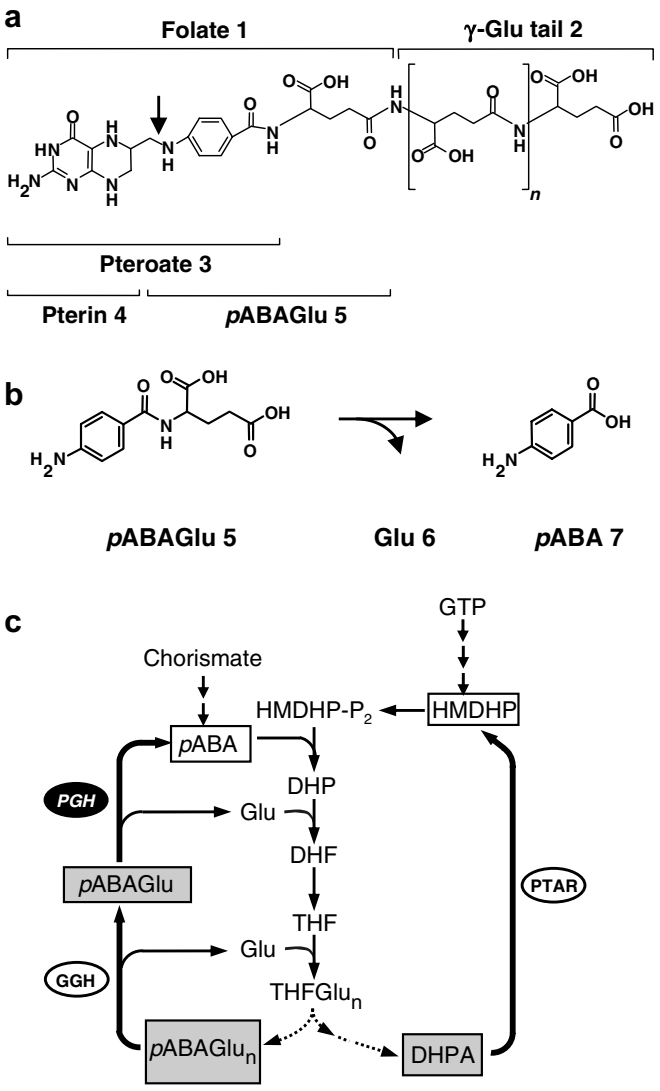

Fig. 1. Structure of folates and hydrolysis of the *p*ABAGlu moiety. (a) Tetrahydrofolate polyglutamate. Polyglutamyl tails of plant folates contain up to about six residues. Oxidative scission of the C9-N10 bond (arrow) yields pterin **4** and *p*ABAGlu **5** fragments. (b) The *p*ABAGlu **5** hydrolysis reaction. (c) Folate salvage reactions (broad arrows) in relation to folate biosynthesis. The *p*ABAGlu **5** fragment from folate **1** cleavage (and its polyglutamyl **2** forms, $pABAGlu_n$) can be recycled for use in folate synthesis after hydrolysis. Removal of the polyglutamyl tail by γ-glutamyl hydrolase (GGH) may be needed before *p*ABAGlu hydrolase (PGH) acts. The pterin **4** fragment (dihydropterin-6-aldehyde, DHPA) from folate cleavage can be recycled to the folate synthesis intermediate hydroxymethyldihydropterin (HMDHP) by the action of pterin aldehyde reductase (PTAR). Other abbreviations: DHP, dihydropteroate; DHF, dihydrofolate; Glu_n, poly-γ-glutamate; THF, tetrahydrofolate; $-P_2$, diphosphate.

of folate **1** breakdown products (Fig. 1c). Although *p*ABAGlu hydrolase (PGH) activity was detected in extracts of *Arabidopsis* and pea (*Pisum sativum* L.) leaves and of tomato (*Lycopersicon esculentum* Mill.) fruit, nothing further is known about this enzyme (Orsomando et al., 2006).

Nor, with one exception, is much known about *p*ABAGlu-hydrolyzing enzymes from other organisms. The only well-characterized protein known to hydrolyze *p*ABAGlu **5** is carboxypeptidase G (CPG, EC 3.4.17.11), a di-zinc enzyme from *Pseudomonas* and other bacteria (McCullough et al., 1971; Albrecht et al., 1978; Sherwood et al., 1985). CPG also cleaves the *p*ABA–glutamate bond in folates and folate analogs, releasing pteroate **3** and glutamate **6** fragments, and can remove the γ-glutamyl tail from polyglutamates by exopeptidase action. The *Pseudomonas* enzyme has been cloned (Minton et al., 1983). Other than CPG, there is genetic evidence that the *Escherichia coli abgA* and *abgB* gene products (which share weak sequence similarity with CPG) have PGH activity (Hussein et al., 1998; Carter et al., 2007). In addition, microorganisms and mammals are known to have an enzyme that releases pteroate from folate (Oe et al., 1983); this enzyme has neither been tested with *p*ABAGlu **5** as substrate nor the encoding gene cloned.

After an initial survey of PGH activity in diverse plants, we determined the subcellular location of PGH activity in pea leaves and fractionated and characterized the activity from *Arabidopsis* leaves and roots. We also screened all *Arabidopsis* proteins with significant sequence similarity to CPG for PGH activity.

2. Results and discussion

2.1. Survey of pABAGlu hydrolase activity

Desalted extracts of various plant tissues were surveyed for PGH activity using a radioassay based on product separation by TLC (see Section 4, assay B). This assay, which uses a subsaturating concentration of [^{14}C]*p*ABAGlu **5** (18–26 μM), is more sensitive and specific than that employed previously (Orsomando et al., 2006). PGH activity was readily detected in all tissues analyzed, which included those tested in a pilot study (Orsomando et al., 2006).

PGH activity was consistently higher in roots than in leaves, the difference ranging from 3- to 80-fold in *Arabidopsis*, pea, and maize (*Zea mays* L.) (Fig. 2). It noteworthy that, for pea at least, this pattern is the inverse of that for folate biosynthesis enzymes, whose protein and RNA levels are about 5-fold lower in roots than in leaves (Jabrin et al., 2003). Roots from *in vitro* cultured *Arabidopsis* plantlets had less activity than those grown hydroponically but were easier to produce and had no microbial contaminants, and so were chosen for subsequent work on roots.

2.2. Subcellular distribution of pABAGlu hydrolase activity

PGH was localized by cellular fractionation and enzyme assay in pea leaves, which – while low in PGH activity – are the tissue of choice for obtaining high yields of intact organelles (Baldet et al., 1993) and have been much used in folate biochemistry (e.g., Chen et al., 1997; Jabrin et al., 2003). Marker enzyme assays con-

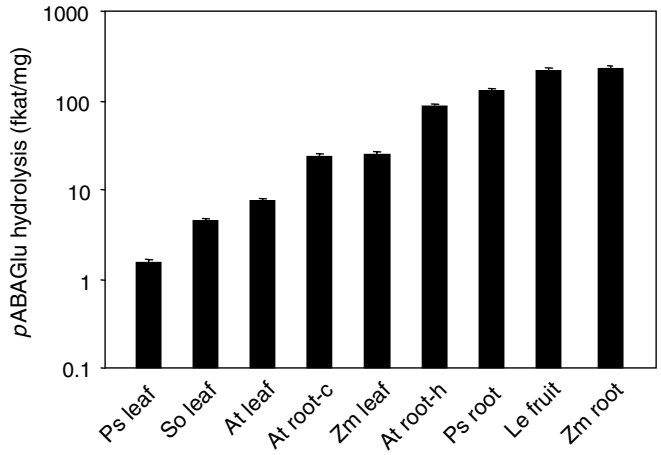

Fig. 2. *p*ABAGlu hydrolase activities in various plant tissues. [^{14}C]*p*ABA **7** production was measured by assay B, with [^{14}C]*p*ABAGlu **5** concentration of 18–26 μM. Pea (Ps), *Arabidopsis* (At) and maize (Zm) leaves were 9, 28, and 11-days-old, respectively. Pea, maize, *Arabidopsis* cultured roots (-c) and hydroponic (-h) roots were 10, 6, 23, and 48-days old, respectively. Tomato (Le) pericarp was in the mature green stage. Spinach (So) leaves were fully expanded. Values are the means of three replicates and SE. Note that the scale is logarithmic.

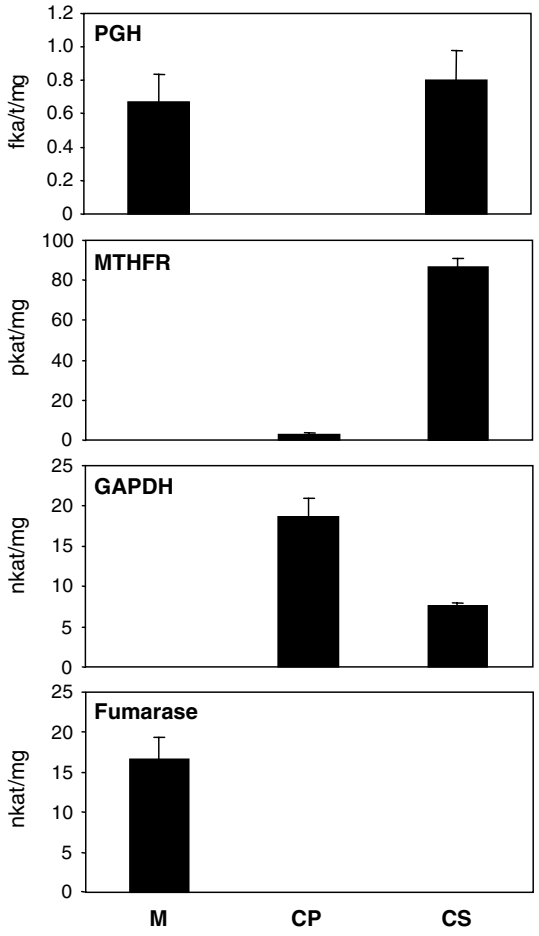

Fig. 3. Localization of *p*ABAGlu hydrolase activity in pea mesophyll cells by subcellular fractionation. Chloroplasts (CP) and mitochondria (M) were purified on Percoll gradients. A fraction enriched in cytosol and vacuole contents (CS) was prepared from pea leaf protoplasts by pelleting intact organelles. The specific activities of PGH and marker enzymes were assayed in each fraction. Markers were: NADP-linked glyceraldehyde-3-phosphate dehydrogenase (GAPDH, chloroplast), fumarase (mitochondrion), and methylenetetrahydrofolate reductase (MTHFR, cytosol). Data are the means and SE of data from three independent preparations of each fraction.

firmed that purified chloroplasts and mitochondria were essentially uncontaminated by other fractions (Fig. 3). PGH activity was detected in mitochondrial and cytosol plus vacuole fractions, but not in chloroplasts (Fig. 3). Multiplying the specific activity of PGH in each fraction by the percentage of cellular protein present in that fraction indicated that 89% of the cellular activity is in the cytosol plus vacuole fraction (Table 1). The PGH activity of vacuolar preparations was not enriched relative to the cytosol/vacuole fraction, although the vacuolar marker α-mannosidase was enriched up to three-fold (not shown). This indicates that PGH is certainly not solely vacuolar but is possibly partially so. In any case, the occurrence of activity in two subcellular fractions signals the presence of at least two PGH isoforms.

2.3. Chromatographic separation of pABAGlu hydrolase activity

To further investigate PGH, activity from *Arabidopsis* leaves and roots was precipitated with $(NH_4)_2SO_4$ and separated by hydrophobic interaction (Octyl-Sepharose) followed by anion exchange (Mono Q) and gel filtration (Superdex 200) steps. The activity from both leaves and roots eluted as one broad peak from Octyl-Sepharose columns (not shown) and also from the Mono Q column, although a shoulder was sometimes evident in the Mono Q peak (Figs. 4 and 5). Subsequent Superdex 200 fractionation yielded a single 200-kDa peak from roots, but three peaks from leaves, with estimated molecular masses of 90, 200, and 360 kDa (Figs. 4 and 5). In some leaf preparations, an additional 40-kDa peak was also present (not shown). While consistent with the existence of various PGH isoforms (i.e., various gene products), these data could also be explained by oligomerization of a single protein.

Table 1
Calculated distribution of *p*ABAGlu hydrolase activity among subcellular fractions of pea leaves

Fraction	PGH activity[a] (fkat/mg)	Protein[b] (% of cell total)	PGH distribution (% of cell total)
Cytosol + vacuole	0.80	26	89
Mitochondria	0.67	4	11
Chloroplasts	0	70	0

[a] Values taken from Fig. 3.
[b] Values from Jabrin et al. (2003).

Representative purifications from leaves and roots are summarized in Table 2. Overall purification was 268-fold from leaves and 86-fold from roots; activity was generally

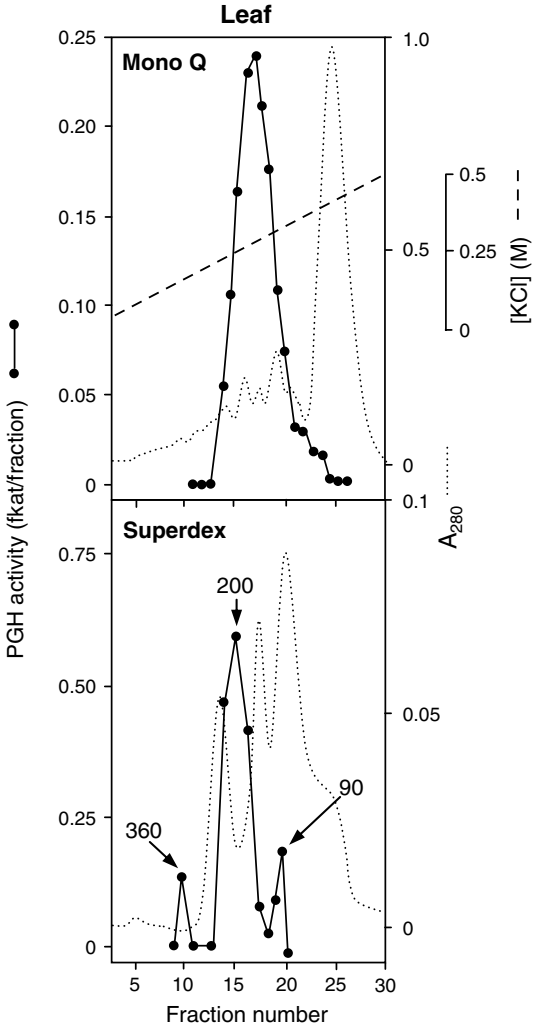

Fig. 4. Elution profiles of *Arabidopsis* leaf *p*ABAGlu hydrolase activity from Mono Q and Superdex 200 columns. After $(NH_4)_2SO_4$ and Octyl-Sepharose steps, activity was applied to a Mono Q column, whose peak fractions were then applied to the Superdex 200 column. Fraction volumes were 300 μL for Mono Q, 320 μL for Superdex 200.

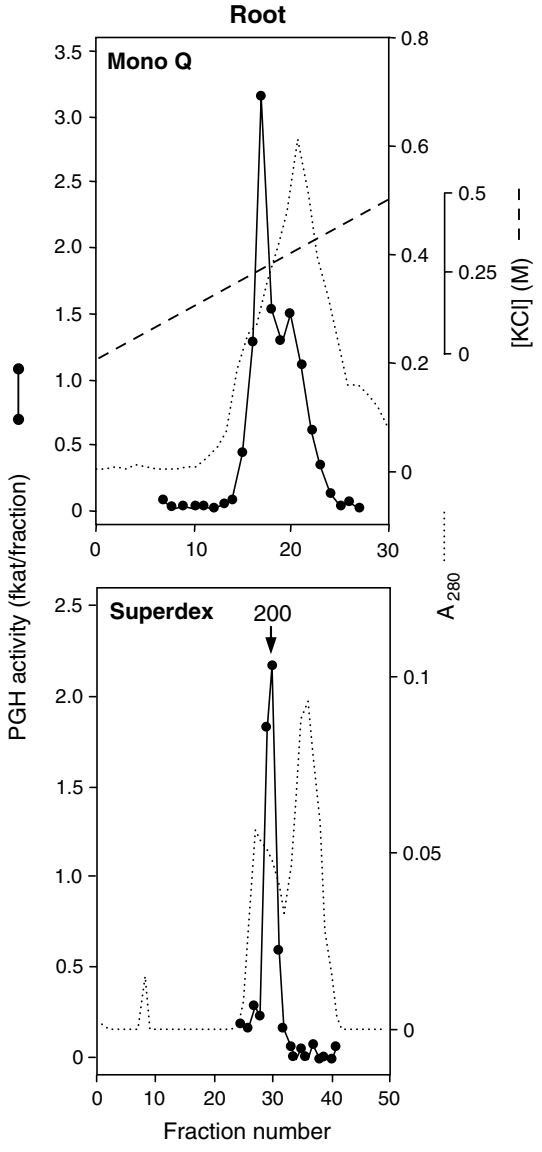

Fig. 5. Elution profiles of *Arabidopsis* root *p*ABAGlu hydrolase activity from Mono Q and Superdex 200 columns. After $(NH_4)_2SO_4$ and Octyl-Sepharose steps, activity was applied to a Mono Q column, whose peak fractions were then applied to the Superdex 200 column. Fraction volumes were 300 μL for Mono Q, 400 μL for Superdex 200.

lost at each column step, and final activity recoveries were only 4–7%. SDS–PAGE analysis of the final products revealed many protein bands (not shown). Attempts at further purification by dye-affinity chromatography resulted in $\geqslant$90% loss of the remaining activity.

PGH enzymes are probably rare proteins, as the following theoretical calculation shows. Taking *Arabidopsis* PGHs to have the same specific activity as pure CPG (3.6–12 μkat/mg) (McCullough et al., 1971; Sherwood et al., 1985) and the activity in cultured roots (Fig. 2), extrapolated to V_{max} (see below), as ~0.4 pkat/mg. then the predicted abundance of PGH proteins is $\leqslant$1 in 10^7.

2.4. Enzymatic properties of root pABAGlu hydrolase

As roots were richer in PGH activity than leaves (Fig. 2) and gave one major peak on Superdex 200, characterization work was done mainly with root PGH using fractions from this peak.

Root PGH was optimally active at pH 7.3–7.5, with half-maximal activity at pH 6.5 and 8.3. Inhibitors were used to test whether the activity is due to a serine-, cysteine-, aspartic-, or metallohydrolase (Table 3). Activity was fairly insensitive to serine, cysteine, and aspartic reagents, but was inhibited totally by one of the metal chelators tested (8-hydroxyquinoline-5-sulfonate) and moderately (53%) by another (TPEN). Moreover, the root enzyme showed modest but significant stimulation by 0.1 mM Mn^{2+} (54%; $P < 0.05$) but not by Zn^{2+} or Ni^{2+}; Cu^{2+} and Hg^{2+} abolished activity. Taken together, these data point to a metalloenzyme. Compounds (1 mM) found

Table 2
Purification of *p*ABAGlu hydrolase from *Arabidopsis* leaves and roots

Step	Total protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Purification (-fold)	Yield (%)
Leaves[a]					
Crude leaf extract	70	0.369	0.0053	1	100
$(NH_4)_2SO_4$ (50–80%)	13	0.063	0.0049	0.9	17
Octyl-Sepharose HiTrap	5.6	0.093	0.0174	3.3	25
Mono Q HR 5/5	0.35	0.051	0.146	28	14
Superdex 200 HR 10/30	0.018	0.026	1.419	268	7
Roots[b]					
Crude root extract	92	5.07	0.055	1	100
$(NH_4)_2SO_4$ (0–80%)	87	6.74	0.089	1.6	133
Octyl-Sepharose 4 Fast Flow	5.1	1.58	0.309	5.6	31
Mono Q HR 5/5	0.38	0.42	1.12	20.2	8
Superdex 200 HR 10/30	0.045	0.21	4.77	86	4

[a] About 30 g of leaves were used. Enzyme activity was measured using assay A and a [^{3}H]*p*ABAGlu concentration of 5 μM.
[b] About 75 g of roots were used. Enzyme activity was measured using assay B and a [^{14}C]*p*ABAGlu concentration of 18.2 μM.

Table 3
Inhibitor sensitivity of *p*ABAGlu hydrolase from *Arabidopsis* roots

Inhibitor	Target	Activity[a] (% of control)
EDTA 1 mM	Metals	79
1,10-Phenanthroline 100 μM	Metals	75
TPEN[b] 100 μM	Metals	47
8-Hydroxyquinoline-5-sulfonate 1 mM	Metals	0
Phenylmethylsulfonylfluoride 1 mM	Serine, cysteine	85
5,5′-Dithio-bis(2-nitrobenzoic acid) 1 mM	Cysteine	75
Pepstatin 1.5 μM	Aspartate	72

[a] Control activity in the absence of inhibitor was 3.6–4.8 pkat/mg, measured at a [^{14}C]*p*ABAGlu concentration of 18.2–25.9 μM using assay B. Values are means of three determinations.
[b] *N*,*N*,*N*′,*N*′-tetrakis[2-pyridylmethyl]ethylenediamine.

to have little (⩽24%) effect on activity were ascorbate, glutathione, β-mercaptoethanol, and ATP (not shown). DTT (1 mM) caused 53% inhibition.

The relation between velocity and *p*ABAGlu concentration was Michaelian for the root enzyme (Fig. 6), with an apparent K_m value of 370 ± 19 μM (mean ± SE of three determinations). Root PGH activity was inhibited by folic acid **1**; measured at a *p*ABAGlu **5** concentration of 19 μM, the concentration of folic acid **1** giving 50% inhibition (IC_{50}) was 52 ± 8 μM (mean ± SE of three determinations). In view of this observation, and because CPG cleaves folates as well as *p*ABAGlu **5** (McCullough et al., 1971), we tested folic acid **1** as a substrate for the root enzyme preparation, using a TLC radioassay (Oe et al., 1983) similar to that used to measure *p*ABAGlu **5** hydrolysis. This assay indicated that folic acid **1** was cleaved to pteroate **3** (Fig. 7a), which was confirmed by HPLC (Fig. 7b). As the root enzyme preparation was not homogeneous, the activity against folic acid **1** cannot necessarily be ascribed to the same protein(s) as PGH activity. This is, however, the most economical explanation.

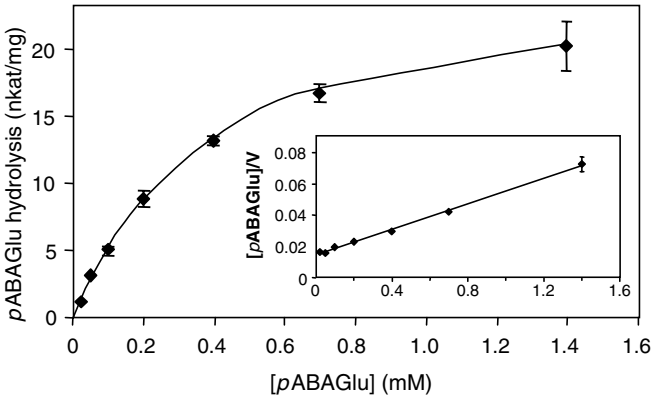

Fig. 6. Kinetic analysis of *Arabidopsis* root *p*ABAGlu hydrolase activity. Activity was measured using assay A, and various concentrations of [^{14}C]*p*ABAGlu **5**. Incubation time was 5 h. The main graph is a velocity versus *p*ABAGlu **5** concentration plot, with mean values (±SE) for three independent experiments; the inset is a Hanes plot for the mean data.

2.5. Carboxypeptidase G-like proteins in Arabidopsis

The only enzyme certainly known to hydrolyze *p*ABAGlu **5** is CPG (McCullough et al., 1971) although genetic evidence indicates that the *E. coli* AbgA and AbgB proteins, which are weakly similar to CPG, also do so (Hussein et al., 1998; Carter et al., 2007). As all three belong to the M20 metallopeptidase family, and our data implicated a metalloenzyme (Table 3), we searched the *Arabidopsis* genome for similar proteins. CPG, AbgA, and AbgB sequences gave significant matches only to auxin conjugate hydrolases (LeClere et al., 2002) and to the At4g17830 protein, a putative metallopeptidase. The auxin conjugate hydrolase family of *Arabidopsis* includes four Mn^{2+}-dependent proteins that cleave indole acetic acid-amino acid conjugates (ILL1, ILL2, ILR1, and IAR3) and two proteins (ILL3 and ILL6) whose substrate is unknown (LeClere et al., 2002).

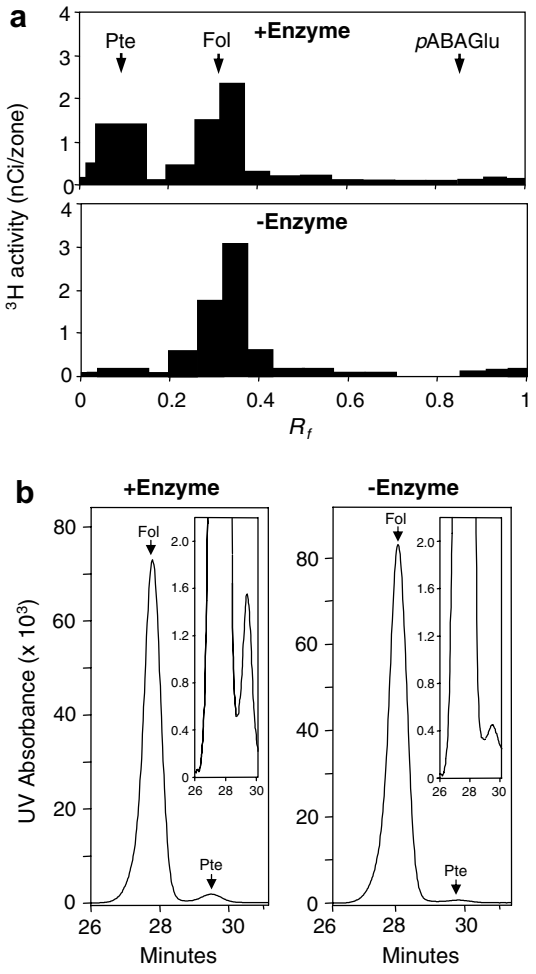

Fig. 7. Evidence for folic acid-hydrolyzing activity in the *p*ABAGlu hydrolase preparation from Arabidopsis roots. (a) TLC separation of radioactivity after incubation of [^{3}H]folic acid **1** (12 nCi, 0.28 pmol) in 10-μL reaction mixtures for 5 h plus or minus enzyme (0.7 μg). Positions of co-chromatographed standards are indicated. Note the absence of label from the *p*ABAGlu zone; this confirms that only the *p*ABA–glutamate bond of folic acid was cleaved, not the pterin–*p*ABA bond. (b) HPLC separation of 10-μL reaction mixtures containing 5 nmol of folic acid **1** after incubation for 5 h plus or minus enzyme (0.18 μg). The inset shows the pteroate **3** peak on a 25-fold magnified UV absorbance scale. Running positions of standards are indicated.

The six members of the auxin conjugate hydrolase group and the At4g17830 protein were expressed in *E. coli* and tested for PGH activity. The former were expressed as GST fusions and purified using glutathione–agarose columns. At4g17830 was expressed with a hexahistidine tag and purified by Ni^{2+} affinity chromatography. The IAA-alanine hydrolyzing activities of two of the most efficient auxin conjugate hydrolases – ILL2 and IAR3 – were used as positive controls, and were found to be similar to those previously reported (Davies et al., 1999; LeClere et al., 2002). Although all seven proteins were enriched to near-homogeneity, none of them had significant PGH activity; only ILR1 showed any trace of activity (Fig. 8).

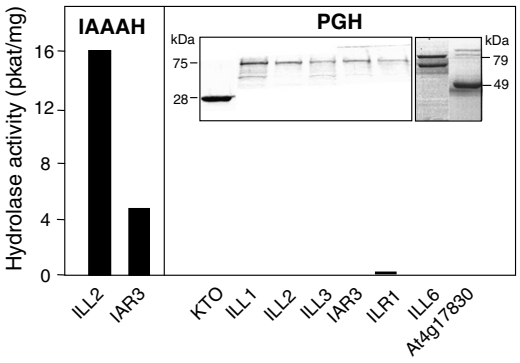

Fig. 8. Screening recombinant *Arabidopsis* CPG-like proteins for PGH activity. The auxin conjugate hydrolase family members were expressed as glutathione *S*-transferase (GST) fusions in pGEX-KTO (KTO); At4g17830 was expressed with a C-terminal histidine tag in pET28b. Enzyme activities were assayed on proteins purified by affinity chromatography. Denaturing gel electrophoresis confirmed the presence of recombinant protein bands (inset) with the expected M_r values (28 kDa for GST; 75–79 kDa for GST–auxin conjugate hydrolase fusions; 49 kDa for At4g17830). PGH activity was measured in the presence of 1 mM $MnCl_2$; results with 1 mM $ZnCl_2$ or with no added divalent cations were the same. As positive controls, ILL2 and IAR3 were tested for indole acetic acid–alanine hydrolase (IAAAH) activity in the presence of 1 mM $MnCl_2$. Data are means of duplicate determinations.

3. Concluding remarks

Our evidence is consistent with the *p*ABAGlu hydrolase activity of both pea and *Arabidopsis* being due to two or more isoforms, at least one of which is a metalloenzyme and all of which are of low abundance. The existence of more than one isoform is presaged by the situation in *E. coli*, in which disrupting *abgA* or *abgB* does not much affect PGH activity (Hussein et al., 1998).

An obvious approach to identifying plant PGH genes – screening all *Arabidopsis* homologs of bacterial PGHs for activity – indicated that none of them is a PGH. From this it follows that plant proteins with PGH activity must be novel in the sense either that they are: (a) known enzymes not yet known to attack *p*ABAGlu **5**, or (b) proteins whose activity is so far unknown. The former possibility seems likelier, for two reasons. First, *p*ABAGlu **5** has never been tested as a substrate for most peptidases, which are good candidates *a priori*. Second, the tyrosine-*p*ABA bond in the synthetic peptide *N*-benzoyl-L-tyrosyl-*p*ABA is cleaved by chymotrypsin (Yamato and Kinoshita, 1978). Given the failure of sequence homology to find plant proteins with PGH activity, this task clearly requires an approach that makes no prior assumptions about the nature of the enzymes, such as functional complementation screening in bacteria. The pilot studies of Hussein et al. (1998) on the *abgA* and *abgB* genes in *E. coli* suggest that this approach may be feasible.

Lastly, our finding that the enzyme preparation from *Arabidopsis* roots can cleave folates is not unprecedented inasmuch as CPG is known to do the same thing (McCullough et al., 1971). It nevertheless emphasizes an often-

overlooked possibility, namely that folate breakdown is in part enzymatic – and hence subject to regulation – as well as merely chemical (Suh et al., 2001).

4. Experimental

4.1. Reagents

[*Ring*-^{14}C]*p*ABA **7** (55 mCi/mmol), [3,5-^{3}H]*p*ABA **7** (26.2 Ci/mmol) and [3′,5′,7,9-^{3}H]folic acid **1** (43.2 Ci/mmol) were from Moravek Biochemicals (Brea, CA, USA). [^{14}C]*p*ABAGlu **5** and [^{3}H]*p*ABAGlu **5** were prepared as described (Orsomando et al., 2006). [^{3}H]Folic acid **1** was purified before use by folate affinity chromatography (Gregory and Toth, 1988). All protein chromatography columns were from GE Healthcare (Piscataway, NJ, USA).

4.2. Plant material

Arabidopsis thaliana (L.) Heynh. ecotype Columbia plants for leaf production were grown at 23–28 °C in 12-h days (photosynthetic photon flux density 80 μE m^{-2} s^{-1}) in potting soil irrigated with water. For root production, plants were grown hydroponically as described (Gibeaut et al., 1997). *Arabidopsis* plantlets were cultured axenically in 0.5× liquid MS salts containing 10 g/L sucrose (Prabhu et al., 1996). Tomato *Lycopersicon esculentum* (cv. Micro-Tom) and pea (*Pisum sativum*) (cv. Laxton's Progress 9), plants were grown as described (Díaz de la Garza et al., 2004; Orsomando et al., 2005). Maize (*Zea mays* cv. NK508) leaves were from 11-day old plants grown in potting soil in a naturally lit greenhouse. Spinach (*Spinacia oleracea*) was purchased locally.

4.3. Subcellular fractionation of pea leaves

Mitochondria and chloroplasts were purified on Percoll gradients (Cline, 1986; Douce et al., 1987). A fraction enriched in cytosol plus vacuole contents, and vacuoles, were prepared from protoplasts (Orsomando et al., 2005). Protoplasts were purified on a three-step sucrose-sorbitol gradient as described (Baldet et al., 1993; Orsomando et al., 2005) except that the middle and bottom layer densities were increased by adding 4% and 13% (v/v) Percoll, respectively. Fractions were supplemented with bovine albumin (1 mg/mL) and concentrated 6- to 13-fold before PGH assays. Marker enzymes were extracted and assayed as described (Orsomando et al., 2005).

4.4. Hydrolase assays

For *p*ABAGlu hydrolase, standard reaction mixtures (final volume 10 μL) contained 8 μL of KPi buffer, 50 mM, pH 7.4 or 8.0, 0.1–16 μg protein), 1 mM $MnCl_2$, and either 10–14 nCi (182–259 pmol) of [^{14}C]*p*ABAGlu **5** or 42-166 nCi (70–280 pmol) of [^{3}H]*p*ABAGlu **5**. Incubation was for 3–8 h at 30 °C. [^{14}C]*p*ABA **7** was separated either by EtOAc partitioning (Orsomando et al., 2006) (assay A) or by TLC (assay B). [^{3}H]*p*ABA **7** was separated by the former procedure. In the latter, reactions were mixed with unlabeled *p*ABA **7** and *p*ABAGlu **5** carriers (100 nmol each) and applied to 1-cm origins on 10-cm silica gel 60 F_{254} TLC plates (Merck, Darmstadt, Germany). After developing with EtOAc:MeOH:H_2O (77:13:10, v/v/v), the *p*ABA zone (R_f 0.9) was scraped out for scintillation counting. [^{14}C]*p*ABA **7** formation was linear with respect to time and amount of enzyme. Routine assays in this study used a subsaturating [^{14}C]*p*ABAGlu **5** concentration, 4.5-fold less than that used previously (Orsomando et al., 2006); the activity values reported are consequently lower. Folate hydrolase activity was assayed by a TLC method (Oe et al., 1983) similar to assay B, using [^{3}H]folic acid **1** as substrate and quantifying pteroate release. The identity of pteroate **3** was confirmed by HPLC with UV detection (300 nm) essentially as described (Díaz de la Garza et al., 2004). Apparent K_m values were determined from Hanes plots (Fig. 6).

4.5. Protein extraction and purification

All steps were at 0–4 °C. Protein was estimated by the dye-binding method (Bradford, 1976). For tests of PGH activity in various plant sources, proteins were extracted and desalted as described (Orsomando et al., 2006). For protein purification, leaves from seven-week old *Arabidopsis* plants or roots from 25- to 28-day-old cultures were triturated in liquid N_2 and suspended (1 g/2.5 mL) in 50 mM KPi, pH 8.0 containing 3% (w/v) polyvinylpolypyrrolidone. The brei was centrifuged (12,000*g*, 20 min) and filtered through Miracloth, with proteins fractionated by adding finely ground $(NH_4)_2SO_4$ to obtain the desired concentration (Table 2). After stirring for 30 min and centrifuging (13,000*g*, 20 min), the pellet was redissolved in 20 mL of 50 mM KPi, pH 8.0, 30% saturated with $(NH_4)_2SO_4$ (Buffer A).

For root proteins, the solution was applied to a 1.3 × 5.6 cm Octyl Sepharose 4 Fast Flow column equilibrated with Buffer A. The column was washed with Buffer A (2.5 mL/min) until the A_{280} of the effluent was <0.02. PGH activity was eluted (2.0 mL/min) with an 83.5-mL linear gradient (100–0% Buffer A/0–100% KPi 50 mM, pH 8.0), collecting 2.5-mL fractions. Fractions with activity were pooled and desalted on PD-10 columns equilibrated with 50 mM KPi, pH 8.0. For leaf proteins, two 0.7 × 2.5 cm Octyl-Sepharose HiTrap columns were coupled and replaced the Fast Flow column.

Active fractions from the Octyl Sepharose step were loaded (0.5 mL/min) onto a Mono Q HR 5/5 column equilibrated with 50 mM KPi, pH 8.0, and the column was washed with this buffer until the A_{280} of the effluent fell to zero. PGH activity was eluted (0.5 mL/min) with a 9-mL linear gradient of 0–0.5 M KCl in 50 mM KPi, pH 8.0, collecting 0.3-mL fractions. Active fractions were

pooled and concentrated to 0.5 mL in a Centricon-10 (Millipore, Billerica, MA, USA).

The concentrate from the Mono Q step was applied (0.25 mL/min) to a Superdex 200 HR 10/30 column equilibrated with 50 mM KPi, pH 8.0 containing 50 mM KCl, and eluted with the same buffer, collecting 0.4-mL fractions. From roots, fractions making up the PGH activity peak were pooled, concentrated to 0.28 mL, brought to 10% (v/v) glycerol, frozen in liquid N_2, and stored at −80 °C until use. To estimate native M_r, the Superdex column was calibrated with carbonic anhydrase (29 kDa), bovine albumin (66 kDa), alcohol dehydrogenase (150 kDa), α-amylase (200 kDa), apoferritin (443 kDa), and bovine thyroglobulin (669 kDa).

4.6. Recombinant protein expression and purification

Constructs of cDNAs of the *Arabidopsis* auxin-conjugate hydrolase family (ILL1, ILL2, ILL3, ILL6, ILR1, and IAR3) in pGEX-KTO (Davies et al., 1999; LeClere et al., 2002) were from B. Bartel and R. Rampey (Rice University, Houston, TX, USA). pGEX-KTO is an expression vector in which the cloned protein is fused to the C-terminus of glutathione *S*-transferase. The At4g17830 cDNA (obtained from the Arabidopsis Biological Resource Center, OH, USA) was modified by using splice overlap extension PCR to ablate an internal NcoI site, and the modified At4g17830 cDNA was cloned between the NcoI and XhoI sites of pET28b (Novagen), which adds a C-terminal hexahistidine tag. Constructs were introduced into *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene), which were grown at 37 °C in LB medium until A_{600} reached 0.6. Temperature was then dropped to 25 °C and isopropyl-D-thiogalactopyransoside was added (final concentration 0.1 mM). Incubation was continued for 3 h at the same temperature. Subsequent procedures were at 0–4 °C. Cells from 50-mL cultures were pelleted, resuspended in 2 mL of 50 mM Tris–HCl, pH 8.0, 0.1 mM $MnCl_2$, 0.01 mM $ZnCl_2$, and broken in a Mini-BeadBeater (Biospec, Bartlesville, OK, USA). Lysates were cleared by centrifugation (10,000*g*, 10 min), desalted on PD10 columns equilibrated with 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3. GST-fusion proteins were purified on GSH-agarose resin and the histidine-tagged protein on Ni-NTA agarose resin (Qiagen, Valencia, CA, USA) according to the manufacturers' recommendations. For extracts of cells expressing ILL2 or IAR3, hydrolysis of indole acetic acid–alanine was measured as described (LeClere et al., 2002).

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