Cloning and Characterization of Mitochondrial

5-Formyltetrahydrofolate Cycloligase from Higher Plants

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Running Title: Plant 5-formyltetrahydrofolate cycloligase
5-Formyltetrahydrofolate cycloligase (5-FCL) catalyzes the conversion of 5-formyltetrahydrofolate (5-CHO-H₄PteGluₙ) to 5,10-methenyltetrahydrofolate and is considered to be the main means whereby 5-CHO-H₄PteGluₙ is metabolized in mammals, yeast, and bacteria. 5-CHO-H₄PteGluₙ is known to occur in plants and to be highly abundant in leaf mitochondria. Genomics-based approaches identified *Arabidopsis* and tomato cDNAs encoding proteins homologous to 5-FCLs of other organisms but containing N-terminal extensions with the features of mitochondrial targeting peptides. These homologs were shown to have 5-FCL activity by characterizing recombinant enzymes produced in *Escherichia coli* and by functional complementation of a yeast *fau₁* mutation with the *Arabidopsis* 5-FCL cDNA. The recombinant *Arabidopsis* enzyme is active as a monomer, prefers the penta- to the monoglutamyl form of 5-CHO-H₄PteGluₙ, and has kinetic properties broadly similar to those of 5-FCLs from other organisms. Enzyme assays and immunoblot analyses indicated that 5-FCL is located predominantly if not exclusively in plant mitochondria and that the mature, active enzyme lacks the putative targeting sequence. Serine hydroxymethyltransferase (SHMT) from plant mitochondria was shown to be inhibited by 5-CHO-H₄PteGluₙ, as are SHMTs from other organisms. Since mitochondrial SHMT is crucial to photorespiration, 5-FCL may help prevent 5-CHO-H₄PteGluₙ from reaching levels that would inhibit this process. Consistent with this possibility, 5-FCL activity was far higher in leaf mitochondria than root mitochondria.
INTRODUCTION

5-Formyltetrahydrofolate cycloligase, EC 6.3.3.2 (5-FCL), also termed 5,10-methenyltetrahydrofolate synthetase, catalyzes the irreversible, ATP-dependent conversion of 5-formyltetrahydrofolate (5-CHO-H4PteGlu) to 5,10-methenyltetrahydrofolate (5,10-CH=H4PteGlu). 5-FCL has been purified from bacteria and mammalian liver (1), and cloned from rabbit (2), human (3), and yeast (4). The native proteins from these species are all monomers of 23-28 kDa (4-6), and the bacterial and mammalian enzymes have been shown to be specific for the 6S form of 5-CHO-H4PteGlu (5, 6). Mammalian 5-FCLs appear to be cytosolic enzymes (2, 7) although a small amount of activity (15% of total) was reported in mitochondria from human liver (8). Yeast 5-FCL is likewise predicted to be cytosolic (4). 5-FCL activity has not been studied in plants (9, 10).

The substrate for 5-FCL, 5-CHO-H4PteGlu, has been found in all organisms investigated so far, including plants (1, 9, 11). Its likely main source is hydrolysis of 5,10-CH=H4PteGlu, catalyzed by a side-reaction of serine hydroxymethyltransferase (SHMT) in the presence of glycine (4, 12), although some spontaneous chemical hydrolysis of 5,10-CH=H4PteGlu in mildly acidic subcellular compartments is also possible (13). Unlike other folate species, 5-CHO-H4PteGlu does not serve as a one-carbon (C1) donor, but is a potent inhibitor of SHMT and several other enzymes of C1 metabolism (1). It is therefore considered that 5-CHO-H4PteGlu is a potential regulator of C1 metabolism, and that its cellular concentration is set by a futile cycle comprising its synthesis by SHMT, and its reconversion to 5,10-CH=H4PteGlu by 5-FCL. Specific roles assigned to this cycle in mammalian cells include regulation of de novo purine synthesis (14), of the flux of C1 units from serine into the folate pool (15), and of homocysteine remethylation (7).

5-CHO-H4PteGlu normally represents only 3-10% of the total cellular folate pool in mammals and yeast (4, 7), whereas values of 14-40% are reported for leaves and other metabolically active
plant tissues (16, 17). Moreover, 5-CHO-H₄PteGluₙ makes up 50% of the mitochondrial folate pool in pea leaves (17), which is much more than in mammalian mitochondria (18-20). The high 5-CHO-H₄PteGluₙ content of leaves and their mitochondria is of special interest given the massive SHMT-mediated glycine → serine fluxes that occur during photorespiration in leaf mitochondria (11) because SHMT could, in these conditions, both form 5-CHO-H₄PteGluₙ and be inhibited by it. Plant mitochondrial SHMT has not, however, been tested for sensitivity to 5-CHO-H₄PteGluₙ (9). 5-CHO-H₄PteGluₙ is abundant in some dormant organs, comprising 70% of total folates in soybean seeds (21) and 85% in Neurospora crassa spores (22), and is metabolized rapidly during germination (22). These data suggest a role as a storage form of folate (1).

Given the evidence that 5-CHO-H₄PteGluₙ is a major folate in plants and their mitochondria, we set out to find whether plants have 5-FCL and, if so, where it is in the cell. Here, we describe the cloning and initial characterization of 5-FCL from Arabidopsis and tomato, and demonstrate that the enzyme is located in mitochondria. We further show that plant mitochondrial SHMT, like SHMTs in other organisms, is inhibited by physiological concentrations of 5-CHO-H₄PteGluₙ.

**EXPERIMENTAL PROCEDURES**

*Chemicals and Reagents*—(6R,6S)-5-CHO-H₄PteGlu₁, (6R)-5-CHO-H₄PteGlu₁, (6S)-5-CHO-H₄PteGlu₁, (6R,6S)-5-CHO-H₄PteGlu₅, and (6R,6S)-tetrahydrofolate (H₄PteGlu₁) were obtained from Schircks Laboratories (Jona, Switzerland). L-[1-¹⁴C]Serine (54 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [¹⁴C]Formaldehyde (55 mCi/mmol) was from NEN Life Science Products. Ni²⁺-nitriloacetic acid superflow resin was from Qiagen. Macerase™ was from Calbiochem, and Cellulase R-10 from Yakult Honsha, Tokyo, Japan.

*Plants and Growth Conditions*—Arabidopsis thaliana plants (ecotype Columbia) were grown for 3 weeks in potting soil at 23°C in 12-h days (photosynthetic photon flux density 80 µE m⁻² s⁻¹).
Pea plants (*Pisum sativum* cv. Laxton's Progress 9) were grown in vermiculite at 20°C in 12-h days (150 \( \mu \text{E m}^{-2} \text{ s}^{-1} \)) for 10 days. Cauliflower (*Brassica oleracea*) was purchased locally.

**Yeast Growth Conditions, Plasmids, and Strains**—Synthetic minimal medium (YMD) contained 0.7% yeast nitrogen base without amino acids (DIFCO Bacto®), 2% glucose, and the following supplements (mg/l) when indicated: \( \ell \)-serine, 375; \( \ell \)-leucine, 30; \( \ell \)-tryptophan, 20; uracil, 20; adenine, 20; and \( \ell \)-methionine, 20. Solid media contained 1.5% agar. Liquid cultures were grown at 30°C in a rotary shaker at 250 rpm. The *S. cerevisiae* strain used was WHY1.3.1 (*faul ade16 ade17 ser1 ura3 trp1 leu2*) (4). The plasmids were pVT103-U (23) and pVT101-U containing the coding sequence of the yeast 5-FCL gene, *FAU1* (4).

**cDNA Constructs and Sequence Analysis**—*Arabidopsis* expressed sequence tag (EST) GenBank™ accession number BE038212 was obtained from H. Bohnert (University of Illinois), and tomato EST AW650167 from Clemson University Genomics Institute. Their inserts were sequenced and subcloned into pET28b (Novagen) with modification, as follows. PCR reactions used *Pfu* DNA polymerase (Stratagene). The missing 5'-terminus of the *Arabidopsis* cDNA was cloned by 5'-RACE using the Gibco BRL kit and the gene-specific primers 5'-CACTACCATCTTC-ACGGTCATT-3' and 5'-GGGCATCAACAGGAGCTGGTT-3'. The cloned 5'-RACE product and the BE038212 cDNA were joined by overlap extension PCR (24). The primers for the 5' region were 5'-AAAAAAA\( T\)CATGAGTTGGAGCTCGCGTCTT-3' (5'-forward) and 5'-ATGTTGCAG-AATCTCAGAGA-3' (5'-internal), and for the 3' region 5'-TCTCTGAGATTCTGCAACAT-3' (3'-internal) and 5'-GTACCTCGAGTCACATGCTCTCGGGTAGC-3' (3'-reverse). The resulting full length cDNA was digested with *BspHI* and *XhoI* and cloned between the *NcoI* and *XhoI* sites of pET28b. An *Arabidopsis* cDNA starting at the second methionine codon was amplified from the BE038212 cDNA using the forward primer 5'-AGCACCACCAGCAAAAAAAAACG-A-3' (for-
ward-Met2) and the 3'-reverse primer above, digested with XhoI, and ligated between the XhoI site and the T4 DNA polymerase-infilled NcoI site of pET28b, to give plasmid At5-FCLn. To add a C-terminal hexahistidine tag, the BE038212 cDNA was amplified using the forward-Met2 primer and the reverse primer 5'-GTACCTCGAGCATGCTCTCGGTAGC-3', digested with Ndel and XhoI, and cloned into the corresponding sites of At5-FCLn. For tomato, a full-length cDNA was amplified using the primers 5'-CCTTTTTCATGACATTCTCGGTAGC-3' (forward) and 5'-CCGCTCGAGTCAGTCGAAAACCTCAGAC-3' (reverse), and a cDNA starting at the second methionine codon was amplified using the same reverse primer and the forward primer 5'-CCTTTTTCATGACACCAGCGAGAGAGA-3'. Both tomato cDNAs were digested with BspHI and XhoI, and cloned into the matching sites of pET28b. This strategy changed the second residue of the full length polypeptide from alanine to threonine. Constructs were electroporated into Escherichia coli DH10B cells and verified by sequencing. Sequences were aligned using Clustal W 1.7 (25). Homology searches were made using BLAST programs (26).

cDNA Expression in E. coli—For protein expression, the above pET28b constructs were electroporated into E. coli BL21 (DE3) cells or, for the histidine-tagged Arabidopsis protein, into BL21-CodonPlus™ (DE3) (Stratagene) cells. Cultures were grown at 37°C in LB medium containing 100 µg/ml kanamycin. When A600 reached 0.6–1, isopropyl-D-thiogalactopyranoside was added to a final concentration of 1 mM, and incubation was continued for 3-4 h at 25°C.

cDNA Expression in Yeast—An Arabidopsis cDNA starting at the second methionine codon was amplified as above using the primers 5'-CGGGATCCATGACACCAGCGAGAA-3' (forward) and 5'-TCACATGCTCTCGGTAGC-3' (reverse), and ligated between the BamHI and PvuII sites of pVT103-U. The sequence-verified construct was introduced into yeast strain WHY1.3.1 as
described (27). After initial selection for uracil prototrophy, colonies were inoculated into appropriately supplemented liquid YMD medium and growth rate was monitored at \(A_{600}\).

**Protein Isolation, Molecular Mass Determination, and Antibodies—** *E. coli* cells from a 50-ml culture were harvested by centrifugation and resuspended in 1 ml of 100 mM Hepes-KOH, pH 7.5, containing 1 mM DTT and 10% glycerol (buffer A). Subsequent operations were at 0–4°C. The resuspended cells were broken by agitation in a Mini-BeadBeater (Biospec Products, Bartlesville, OK) using 0.1-mm zirconia/silica beads. The beads were washed with 1 ml of buffer A, and the pooled extract plus wash was cleared by centrifugation (16,000 x g, 20 min) and desalted on a PD-10 column (Amersham Pharmacia Biotech) equilibrated in buffer A. Extracts were frozen in liquid N\(_2\) and stored at –80°C; this did not affect 5-FCL activity. Protein was determined by Bradford's method (28) using bovine serum albumin as standard. Native molecular mass was estimated using a Waters 626 high performance liquid chromatography system equipped with a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech). The reference proteins were cytochrome c, carbonic anhydrase, bovine serum albumin, and \(\beta\)-amylase. Histidine-tagged *Arabidopsis* 5-FCL was purified by affinity chromatography on Ni\(^{2+}\)-nitriloacetic acid resin according to manufacturer's protocols. For kinetic analyses, the protein was purified under native conditions. The 80 mM imidazole eluate (the most homogeneous 5-FCL fraction) was desalted on a PD-10 column equilibrated with 100 mM MES, pH 6.0, containing 10% glycerol, frozen in liquid N\(_2\) and stored at –80°C until use. Freezing did not affect the enzyme activity. For antibody production in rabbits, 5-FCL was purified under denaturing conditions. The pH 5.9 eluate (richest in protein) was desalted twice on PD-10 columns equilibrated with 0.1X phosphate buffered saline (29) and concentrated 10-fold in vacuo. The concentrate was made 0.5% in SDS to resolubilize precipitated protein, and sent to Cocalico Biologicals (Reamstown, PA).
5-FCL Assays—5-FCL activity was measured by discontinuous or continuous spectrophotometric assays (30, 31). Unless otherwise noted, substrates were saturating and product formation was proportional to enzyme concentration and time. All assays were in a final volume of 100 µl, and contained 10 mM β-mercaptoethanol and 0.25% (v/v) Tween-20. Discontinuous assays were run at 30°C in 100 mM buffer containing 2 mM ATP, 4 mM Mg acetate and 1 mM (6R,6S)-5-CHO-H₄PteGlu₁₁, (6R)-5-CHO-H₄PteGlu₁₁, (6S)-5-CHO-H₄PteGlu₁₁, or (6R,6S)-5-CHO-H₄PteGlu₁₅. Buffers were: Mes-KOH, pH 5.5-6.5; Pipes-KOH, pH 6.5-7.5; Heps-KOH, pH 7.0-8.0; or Bicine-KOH, pH 7.5-9.0. Reactions were stopped by adding 200 µl of saturated (NH₄)₂SO₄ solution adjusted with citric acid to pH 3.5 (or, for assays in Bicine, pH 3.0), and centrifuged to clear. The heating step (30) was omitted. 5,10-CH=H₄PteGluᵩ was estimated from absorbance at 350 nm relative to a blank to which ATP was added after incubation; an extinction coefficient of 24,900 M⁻¹ cm⁻¹ was used (32). Continuous assays were run at 23°C in 50 mM Mes-KOH, pH 6.0, using the ATP, Mg acetate, and 5-CHO-H₄PteGluᵩ concentrations indicated; [Mg·ATP] was calculated as described (33). Enzymatic conversion of 5,10-CH=H₄PteGluᵩ to 10-formyl-H₄PteGluᵩ in these conditions was shown to be negligible by monitoring the reaction at 310 nm as well as 360 nm (31). \( K_m \) values were calculated from Hanes plots.

Preparation of Subcellular Fractions—Pea and cauliflower mitochondria and pea chloroplasts were isolated and purified on Percoll gradients as described (34, 35), resuspended in 10 mM K-phosphate, pH 7.5, containing 1 mM serine, 5 mM β-mercaptoethanol, 1 mM EGTA, and 20 µM pyridoxal 5'-phosphate, and broken by 4-5 cycles of freezing and thawing. Organellar extracts were cleared by centrifugation (15 min, 16,000 x g). A cytosolic fraction was prepared from pea leaf protoplasts as described (36). The chloroplast stromal marker NADP-linked glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the mitochondrial matrix marker fumarase were ass-
ayed as described (37); fumarase was inactivated by freezing in liquid N$_2$ and so was assayed in fresh samples. Other activities were measured after freezing in liquid N$_2$ and storing at $-80^\circ$C.

**Protein and Immunoblot Analyses**—Proteins were precipitated with 10% trichloroacetic acid, resuspended in 2x Laemmli buffer at 10 $\mu$g/$\mu$l, and separated by SDS-PAGE on 12.5% gels. Electrophoresis and immunoblotting procedures were as described (38); the *Arabidopsis* 5-FCL antiserum was diluted 1:1000. Recombinant *Arabidopsis* 5-FCL protein in *E. coli* extracts was quantified using an Alphalmager$^\text{TM}$ digital imaging system (San Leandro, CA) by lane density analysis of Coomassie-stained SDS-PAGE gels or by densitometry of immunoblots. For immunoblot densitometry, a calibration curve was prepared using known amounts of the recombinant *Arabidopsis* 5-FCL version lacking the N-terminal region upstream of the second methionine, and with no histidine tag. Purified histidine-tagged protein was not used for calibration because some antibodies in the serum were shown to recognize the tag.

**SHMT Assay**—SHMT activity was assayed at 30$^\circ$C as described (39), with minor modifications. Reactions (100 $\mu$l final volume) were run for 20 min in 50 mM K-phosphate, pH 7.5 containing 1 mM L-[3-$^{14}$C]serine (1 nCi/nmol), 200 $\mu$M H$_4$PteGlu$_1$, 4 mM $\beta$-mercaptoethanol and 250 $\mu$M pyridoxal 5'-phosphate. Reactions were stopped by adding 75 $\mu$l of 1 M Na acetate, pH 4.5, 50 $\mu$l of 100 mM formaldehyde, and 75 $\mu$l of 400 mM dimedone in 50% ethanol, and boiled for 5 min. The labeled product was extracted with 1 ml toluene and a 0.8-ml aliquot was taken for scintillation counting. Data were corrected for recovery of $[^{14}$C]formaldehyde spikes, which was $>85\%$.

**RESULTS**

**Genomics-based Cloning of 5-FCL cDNAs from Arabidopsis and Tomato**—BLAST searches of the *Arabidopsis* database using the protein sequence of human 5-FCL detected a single gene (At5g13050) encoding a putative 5-FCL polypeptide, and three cognate ESTs. Sequencing of the
longest EST (Genbank™ BE038212) showed that it lacks the first 87 nucleotides of the coding sequence. The missing region was cloned by 5'-RACE, and the full-length coding sequence was reconstituted by overlap extension PCR. Southern analysis using the BE038212 cDNA as probe confirmed that there is only one copy of the 5-FCL gene in the Arabidopsis genome (not shown).

BLAST searches of plant EST databases using the Arabidopsis protein sequence detected a single tomato contig (comprising eleven ESTs) encoding a 5-FCL homolog, and >50 similar ESTs from other plants (six dicots, four monocots, a gymnosperm, and a moss). That the tomato ESTs all belong to one contig indicates that tomato, like Arabidopsis, has only one 5-FCL gene. The longest tomato EST (AW650167) was sequenced and shown to encode a full-length polypeptide.

The deduced Arabidopsis and tomato proteins share 23-29% identity with human, yeast, and bacterial 5-FCLs, but are distinct in having N-terminal extensions of at least 40 residues (Fig. 1A). These presequences have the features of mitochondrial targeting peptides (40) and appear to be general in plants because similar extensions are specified by ESTs from other dicots and monocots. Despite their mitochondrial-type presequences, plant 5-FCLs cluster phylogenetically with fungal and animal 5-FCLs, which are known or presumed to be cytosolic, and not with prokaryotic 5-FCLs – including that of Rickettsia, the closest living relative of mitochondria (41) (Fig. 1B). Plant 5-FCLs therefore presumably share a common ancestor with other eukaryotic 5-FCLs.

Complementation of a Yeast Δfaul Mutant by Arabidopsis 5-FCL—Disruption of the yeast 5-FCL gene (FAU1) in a strain in which the purine synthesis genes ADE16 and ADE17 are also disrupted results in a methionine deficiency that reduces the growth rate two- to three-fold (4). The triple mutant Δade16 Δade17 Δfaul was accordingly used as the host for functional complementation tests with an Arabidopsis 5-FCL engineered to remove the putative targeting peptide
(Fig. 1A). This construct decreased the doubling time almost as effectively as the native \textit{FAU1} gene (Fig. 2A), indicating that the protein shorn of its N-terminal extension has 5-FCL activity.

\textit{Expression of Arabidopsis and Tomato 5-FCLs in E. coli}—Full-length 5-FCLs from \textit{Arabidopsis} and tomato were expressed in \textit{E. coli}, together with versions engineered to remove the putative targeting peptide (Fig. 1A). High 5-FCL activity (up to 3 \mu mol min\(^{-1}\) mg\(^{-1}\) protein) was found in desalted extracts of cells harboring each of the four constructs (Fig. 2B). The endogenous activity of the host cells was barely detectable (1.3 nmol min\(^{-1}\) mg\(^{-1}\) protein). Based on this result and the complementation test, the \textit{Arabidopsis} and tomato proteins were designated AtFCL and LeFCL, respectively, and their N-terminally truncated versions were designated AtFCL-n and LeFCL-n.

For both \textit{Arabidopsis} and tomato, the truncated enzyme construct gave higher 5-FCL activity than the full-length one (Fig. 2B). SDS-PAGE analysis of the cell extracts showed that this difference was due largely if not solely to greater expression of the truncated proteins, AtFCL-n and LeFCL-n (Fig. 2C). The estimated molecular masses of AtFCL-n and LeFCL-n were 31 kDa (Fig. 2C), which is \~4 kDa greater than the masses predicted from their sequences. Similar discrepancies have been reported for mammalian 5-FCLs (2, 7).

The activity given by the full-length constructs (Fig. 2B) was at first sight intriguing as it suggested that plant FCLs do not need to have their presequences removed in order to be enzymatically active. However, immunoblot analysis of \textit{E. coli} extracts expressing the full-length \textit{Arabidopsis} protein revealed two bands (33 and 31 kDa), of which the latter was stronger and migrated with AtFCL-n (Fig. 3A). Moreover, by quantifying the 5-FCL protein bands and calculating specific activities, we found that the specific activity of the full-length construct, when based on the amount of 31-kDa protein, was near that of AtFCL-n (Fig. 3B). The activity given by the full-length construct is therefore most likely due to production of a truncated protein in \textit{E. coli} cells.
This truncated product was not further investigated, but may have come from adventitious translation initiation at the second methionine codon (Fig. 1A), i.e., at the same methionine as the engineered truncated protein AtFCL-n.

**Characterization of AtFCL-n**—Genomic evidence, studies of the recombinant enzyme (above), and immunoblot analysis of the protein expressed *in planta* (below) all indicate that the physiologically relevant form of 5-FCL has no N-terminal extension. The truncated *Arabidopsis* protein AtFCL-n was therefore used to measure $K_m$ values, pH dependence, and native molecular mass.

Kinetic constants were determined with histidine-tagged AtFCL-n purified to near-homogeneity by Ni$^{2+}$ chelate affinity chromatography (Fig. 4). Preliminary experiments with crude *E. coli* extracts using 5-CHO-H$_4$PteGlu$_1$ as substrate established that the tag does not affect the kinetic properties of AtFCL-n. The $K_m$ values for all substrates tested (Table I) fall within the range reported for the mammalian, yeast, and *Lactobacillus casei* enzymes (4, 5, 30, 42, 43). Like other 5-FCLs, AtFCL-n prefers the penta- to the monoglutamyl form of 5-CHO-H$_4$PteGlu$_n$, and is specific for the 6$S$ isomer since activity with the 6$R$ form was <5% of that with 6$S$, and the $K_m$ for the 6$S$ form was about half that for the 6$R$,6$S$ racemate (Table I). The turnover number ($k_{cat}$) of AtFCL-n with 5-CHO-H$_4$PteGlu$_1$ as substrate can be estimated from the data of Table I as ~320 min$^{-1}$. This is about three-fold lower than the $k_{cat}$ of human 5-FCL (1000 min$^{-1}$, the highest reported) and about four-fold higher than that of *L. casei* (the lowest) (5, 30). AtFCL-n activity rose steadily between pH 5.5 and 9 (Fig. 3C). This contrasts with mammalian 5-FCLs, which show optima at pH 6.5 or below (30, 32).

The molecular mass of native AtFCL-n was estimated by size exclusion chromatography. The enzyme activity migrated as a symmetrical peak with an apparent mass of 27 kDa, which coin-
cides with the value predicted from amino acid sequence (26.5 kDa) and so is consistent with a monomeric structure. The FCLs from mammals and L. casei are also monomeric (5, 42, 43).

*Localization of 5-FCL in Plant Mitochondria*—The molecular mass and subcellular localization of plant 5-FCLs were investigated by cell fractionation in conjunction with immunoblotting and enzyme assays. Since the recovery of intact organelles from *Arabidopsis* was not efficient, pea leaves, pea roots, and cauliflower florets were used instead. These tissues are well suited for recovery of intact organelles (34-36), and cauliflower is closely related to *Arabidopsis*, whose 5-FCL was used to raise antibodies.

Fractionation of pea leaf cells showed that 5-FCL activity was high only in mitochondria (Fig. 5A). The low 5-FCL activities in cytosol and chloroplast fractions were within the range likely to be caused by cross-contamination by mitochondrial proteins, as shown by the distribution of the fumarase marker (Fig. 5A). These data indicate that 5-FCL is a mitochondrial enzyme in pea leaf cells, and that if other compartments have any activity, it does not exceed a few percent of the total. Consistent with a mitochondrial location, purified mitochondria from cauliflower florets and pea roots also showed 5-FCL activity, but the root activity was low (Fig. 5A).

The matrix of purified cauliflower mitochondria contained a polypeptide that cross-reacted strongly with antibodies to *Arabidopsis* 5-FCL and migrated with the truncated recombinant protein AtFCL-n (Fig. 5B). This result indicates that the ~40-residue presequence has – as expected – been removed from the mature mitochondrial polypeptide.

*Inhibition of SHMT from Pea Leaf Mitochondria by 5-CHO-H_{4}PteGlu_{n}*—Since pea leaf mitochondria are rich in 5-CHO-H_{4}PteGlu_{n} (17) and have high 5-FCL activity (Fig. 5) we tested whether their SHMT is inhibited by 5-CHO-H_{4}PteGlu_{n}, like SHMTs from other organisms. SHMT
activity was measured using subsaturating concentrations of serine and H₄PteGlu₁ to mimic the probable *in organello* conditions (17, 44, 45). Activity was inhibited moderately by (6R,6S)-5-CHO-H₄PteGlu₁ and strongly by (6R,6S)-5-CHO-H₄PteGlu₅ (IC₅₀ ~50 µM) (Fig. 6). This inhibition is likely to be physiologically relevant because 5-CHO-H₄PteGluₙ concentrations in pea leaf mitochondria can be estimated to be 250–500 µM (17, 44), and penta- and tetraglutamyl forms predominate (45).

**DISCUSSION**

The work presented here demonstrates that plants have 5-FCL enzymes, that plant 5-FCL poly-peptides have mitochondrial targeting sequences, and that the active enzyme is located primarily if not solely in mitochondria. Even though plant 5-FCLs are structurally similar to those of other organisms, and have broadly similar kinetic characteristics, mitochondrial localization sets them apart from their cytosolic counterparts in other eukaryotes. In being located in mitochondria in plants but not other eukaryotes, 5-FCL resembles the last five enzymes of *de novo* folate synthesis (11, 46) and, more generally, adds to the growing list of unique features of plant C₁ metabolism (10). Although our biochemical data do not exclude there being a few percent of the total 5-FCL activity in other compartments, genomic data show that *Arabidopsis* has only one 5-FCL gene, and EST data suggest that the same is true of tomato. If there is any extramitochondrial 5-FCL activity in plants, it would therefore have to arise by a dual-targeting mechanism (47).

Green leaf mitochondria have much higher SHMT concentrations than mitochondria from other organs (48). Since leaf mitochondria receive a large photorespiratory influx of glycine during illumination (11), and glycine promotes 5-CHO-H₄PteGluₙ formation *via* SHMT (1), the combination of high titers of both SHMT and glycine may result in a much greater rate of 5-CHO-H₄PteGluₙ formation in leaf mitochondria than in mitochondria of other organs. In this connect-
ion it is noteworthy that our data showed that leaf mitochondria have far higher 5-FCL activity than root mitochondria. Because plant mitochondrial SHMT, like other SHMTs, is inhibited by physiological levels of 5-CHO-H₄PteGlu₅, the strict control of mitochondrial 5-CHO-H₄PteGlu₅ concentration may be essential to photorespiration, and 5-FCL could contribute to this control. This hypothesis is illustrated in Fig. 7.

The mitochondrial localization of plant 5-FCL raises the question of whether its substrate 5-CHO-H₄PteGlu₅ is also solely mitochondrial, and if not, how 5-CHO-H₄PteGlu₅ is metabolized in other cellular compartments. Analysis of folate pools of pea leaves indicates that 5-CHO-H₄PteGlu₅ dominates the mitochondrial folate pool but occurs elsewhere in the cell (17), most probably mainly in the cytosol, which contains the bulk of cellular folates (17, 44). Furthermore, SHMT – the enzyme that produces 5-CHO-H₄PteGlu₅ (4, 12) – occurs in the cytosol and chloroplasts as well as in mitochondria, the distribution in green leaves being roughly 25% cytosolic, 25% chloroplastic, and 50% mitochondrial (48). Plant cells may also generate 5-CHO-H₄PteGlu₅ by chemical hydrolysis of 5,10-CH=H₄PteGlu₅ in the acidic central vacuole (13).

One possibility for the disposal of extramitochondrial 5-CHO-H₄PteGlu₅ in plants is that it is imported into mitochondria and, in support of this, there is a homolog of the mammalian mitochondrial folate transporter in the Arabidopsis genome (46). In addition, at least the C₁ moiety of exogenously supplied 5-CHO-H₄PteGlu₅ can enter the mitochondria in Arabidopsis (49). However, there may be other ways to dispose of 5-CHO-H₄PteGlu₅, perhaps leading to non-folate products. In this context it is interesting that knocking out 5-FCL in yeast gave only a modest (three-fold) increase in 5-CHO-H₄PteGlu₅, and did not affect growth (4). Moreover, ferritin has been found to mediate the oxidative cleavage of 5-CHO-H₄PteGlu₅ in vitro and in vivo (50). Thus, while 5-FCL may be the only enzyme in plants that can recycle 5-CHO-H₄PteGlu₅ back to the metabolically active folate pool, it may not be the only one able to destroy it.
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REFERENCES


FOOTNOTES

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The nucleotide sequence(s) reported in this paper has been submitted to GenBank™/EBI Data Bank with accession number(s) AF516365 (Arabidopsis thaliana 5-FCL), and AF516366 (Lycopersicon esculentum 5-FCL).

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The abbreviations used are: 5-FCL, 5-formyltetrahydrofolate cycloligase; 5-CHO-H₄PteGluₙ, 5-formyltetrahydrofolate; 5,10-CH=H₄PteGluₙ, 5,10-methenyltetrahydrofolate; SHMT, serine hydroxymethyltransferase; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; Mes, 4-morpholineethanesulfonic acid; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); Bicine, N,N-bis(2-hydroxyethyl)glycine.
FIGURE LEGENDS

FIG. 1. Relationship of Arabidopsis and tomato 5-FCL homologs to 5-FCLs of other species. 
A, Alignment of the deduced protein sequences of 5-FCLs from Arabidopsis (At), tomato (Le), Homo sapiens (Hs, GenBank™ AAC41945), Saccharomyces cerevisiae (Sc, S50686), and Escherichia coli (Ec, AAC75949). Identical residues are shaded in black, similar residues in gray. Dashes are gaps introduced to maximize alignment. The triangle marks the methionines in the Arabidopsis and tomato sequences that are the first residues in the truncated recombinant proteins AtFCL-n and LeFCL-n. B, Molecular phylogenetic tree of plant, animal, fungal, and bacterial 5-FCL protein sequences; the circled zone demarcates the eukaryotic sequences. The tree was constructed using Phylip algorithms at the Institut Pasteur server (http://bioweb.pasteur.fr). Sequences were processed using protpars, and a consensus tree was calculated using consense. Bootstrap analysis was performed on 1000 replicates, with the Chlamydophila sequence as out-group. Values by each branch are the number of times that the partition of the sequences into the two sets that are separated by that branch occurred among the trees.

FIG. 2. Evidence that Arabidopsis and tomato 5-FCL homologs have 5-FCL activity. A, Functional complementation of a yeast Δfau1 mutant by AtFCL-n. Growth rates were measured in minimal medium minus or plus methionine for strain WHY1.3.1 (Δade16Δade17Δfau1) transformed with pVT103-U (Vector), with pVT101-U containing the yeast FAU1 gene (+FAU1), or with pVT103-U containing AtFCL-n (+FCL). Doubling times are means for three replicates and S.E. B, 5-FCL activity in extracts of E. coli cells transformed with pET28b alone (pET) or carrying full-length (FL) or truncated (-N) versions of Arabidopsis (Ara) and tomato (Tom) 5-FCLs. Activity was measured in continuous spectrophotometric assays containing 2 mM 5-CHO-H4Pte-Glu1, 2 mM ATP, and 20 mM Mg acetate; data are means of three replicates and S.E. C, Coom-
assie-stained SDS-PAGE gel showing the relative amounts of 5-FCL polypeptides in the *E. coli* extracts used for enzyme assays. The positions of molecular mass standards (kDa) are marked.

**FIG. 3. Characterization of recombinant *Arabidopsis* 5-FCLs.** *A*, Immunoblot analysis of 5-FCL polypeptides in extracts of *E. coli* cells harboring plasmids encoding truncated (-N) and full length (FL) *Arabidopsis* 5-FCLs. Positions of molecular mass standards (kDa) are marked. *B*, Specific activities of the enzymes specified by truncated and full length *Arabidopsis* 5-FCL constructs. 5-FCL polypeptides were quantified as in *Experimental Procedures*; for the full-length construct, the amount of the shorter polypeptide was used to calculate specific activity. 5-FCL activity was measured using continuous assays as in Fig. 2B, using 5-CHO-H₄PteGlu₁ (*Glu₁*) or 5-CHO-H₄PteGlu₅ (*Glu₅*). *C*, Dependence of 5-FCL activity on pH. Activity was measured using the discontinuous assay and is expressed per unit of total protein in the bacterial extract.

**FIG. 4. Purification of histidine-tagged At5-FCL-n under native conditions.** SDS-polyacrylamide gel electrophoresis of histidine-tagged At5-FCL-n isolated by Ni²⁺ chelate affinity chromatography. The gel was stained with Coomassie Blue. *Lane 1* was loaded with 5 µg of crude extract, *lane 2* with 1.5 µg of the fraction not bound by the resin, and *lane 3* with 1.5 µg of purified protein. The positions of molecular markers are indicated.

**FIG. 5. Localization and molecular mass of 5-FCL in plant mitochondria.** *A*, Activities of 5-FCL and marker enzymes in the cytosolic fraction of pea leaf protoplasts (*CS*), Percoll-purified pea chloroplasts (*CP*), and Percoll-purified mitochondria from pea leaves (*LM*), pea roots (*RM*), and cauliflower florets (*CM*). 5-FCL activity was measured using the discontinuous assay as in Fig. 2B. Data are means of three replicates and S.E. *B*, Immunoblot of matrix proteins from Percoll-purified cauliflower floret mitochondria (*CM*; 50 µg) and recombinant, truncated *Arabidopsis* 5-FCL (-N; 20 ng).
FIG. 6. Inhibition of mitochondrial SHMT by 5-CHO-H₄PteGlu₅. SHMT activity in extracts of Percoll-purified pea leaf mitochondria was measured using 1 mM serine and 200 µM (6R,6S)-H₄PteGlu₁, in the presence of various concentrations of (6R,6S)-5-CHO-H₄PteGlu₁ or (6R,6S)-5-CHO-H₄PteGlu₅.

FIG. 7. Scheme showing the photorespiratory glycine → serine flux in leaf mitochondria in the light, and its possible relationship to the metabolism of 5-CHO-H₄PteGlu₅. Note that glycine is required for the SHMT-catalyzed conversion of 5,10-CH=H₄PteGlu₅ to 5-CHO-H₄PteGlu₅ (dotted arrow), and that 5-CHO-H₄PteGlu₅ inhibits SHMT (dashed arrow). By recycling 5-CHO-H₄PteGlu₅ to 5,10-CH=H₄PteGlu₅, 5-FCL may help prevent inhibition of SHMT and so assure unimpaired serine synthesis. GDC, glycine decarboxylase complex.
5-FCL activity was measured using histidine-tagged At5-FCL-n purified under native conditions, and the continuous spectrophotometric assay at 23°C and pH 6.0. Apparent $K_m$ values were calculated from Hanes plots. The values for 5-CHO-H$_4$PteGlu$_n$ substrates were estimated using 500 µM ATP; the value for ATP was estimated using 500 µM (6R,6S)-5-CHO-H$_4$PteGlu$_1$. The Mg acetate concentration was 10 mM. Data are means of measurements with three independent enzyme preparations ± S.E.

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<tr>
<th>Substrate</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
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<tbody>
<tr>
<td>(6R,6S)-5-CHO-H$_4$PteGlu$_1$</td>
<td>41.3 ±2.5</td>
<td>12.1 ±0.5</td>
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<tr>
<td>(6R,6S)-5-CHO-H$_4$PteGlu$_5$</td>
<td>5.8 ±0.5</td>
<td>11.5 ±0.2</td>
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<td>(6S)-5-CHO-H$_4$PteGlu$_1$</td>
<td>17.3 ±0.7</td>
<td>12.6 ±0.3</td>
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<tr>
<td>Mg•ATP</td>
<td>35.2 ±1.09</td>
<td>--</td>
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Figure 7
Cloning and characterization of mitochondrial 5-formyltetrahydrofolate cycloligase from higher plants
Sanja Roje, Machhindra T. Janave, Michael J. Ziemak and Andrew D. Hanson

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