5-Formyltetrahydrofolate Is an Inhibitory but Well Tolerated Metabolite in Arabidopsis Leaves*

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5-Formyltetrahydrofolate (5-CHO-THF) is formed via a second catalytic activity of serine hydroxymethyltransferase (SHMT) and strongly inhibits SHMT and other folate-dependent enzymes in vitro. The only enzyme known to metabolize 5-CHO-THF is 5-CHO-THF cycloligase (5-FCL), which catalyzes its conversion to 5,10-methylenetetrahydrofolate. Because 5-FCL is mitochondrial in plants and mitochondrial SHMT is central to photorespiration, we examined the impact of an insertional mutation in the Arabidopsis 5-FCL gene (At5g13050) under photorespiratory (30 and 370 μmol of CO₂ mol⁻¹) and non-photorespiratory (3200 μmol of CO₂ mol⁻¹) conditions. The mutation had only mild visible effects at 370 μmol of CO₂ mol⁻¹, reducing growth rate by ~20% and delaying flowering by 1 week. However, the mutation doubled leaf 5-CHO-THF level under all conditions and, under photorespiratory conditions, quadrupled the pool of 10-formyl-5,10-methylenetetrahydrofolates (which could not be distinguished analytically). At 370 μmol of CO₂ mol⁻¹, the mitochondrial 5-CHO-THF pool was 8-fold larger in the mutant and contained most of the 5-CHO-THF in the leaf. In contrast, the buildup of 10-formyl-5,10-methylenetetrahydrofolicotates was extramitochondrial. In photorespiratory conditions, leaf glycine levels were up to 46-fold higher in the mutant than in the wild type. Furthermore, when leaves were supplied with 5-CHO-THF, glycine accumulated in both wild type and mutant. These data establish that 5-CHO-THF is an inhibitory metabolite, which it achieves by catalyzing irreversible, ATP-dependent conversion to 5,10-CH₂-THF (SHMT) in the presence of glycine (1, 2). Spontaneous chemical hydrolysis of 5,10-CH₂-THF may be a minor additional source (3). 5-CHO-THF is the most stable natural folate and the most enigmatic, for it is the only one that does not serve as a cofactor in one-carbon metabolism. Instead, 5-CHO-THF is a potent inhibitor of SHMT and most other folate-dependent enzymes in vitro (4, 5). 5-CHO-THF probably acts as a stable storage form of folate in seeds and fungal spores (5–7), but it is not clear what role, if any, it plays in metabolically active tissues (8).

This question is particularly pertinent for leaves. Leaf mitochondria have very high levels of SHMT and, during photorespiration, receive a massive influx of glycine (which leads to a matching SHMT-mediated glycine → serine flux) (9). Conditions in leaf mitochondria therefore favor 5-CHO-THF formation (Fig. 1). Indeed, 5-CHO-THF can comprise 50% of the folate pool in leaf mitochondria (10, 11), which is far more than in mammalian mitochondria (12–14). Furthermore, 5-CHO-THF is reported to make up 14–40% of the folate pool in leaves and other metabolically active plant organs (10, 15), a much higher proportion than the 3–10% typical of mammals and yeast (2, 16).

5-Formyltetrahydrofolate cycloligase, EC 6.3.3.2 (5-FCL, also known as 5,10-methylenetetrahydrofolate synthetase), is the only enzyme known to recycle 5-CHO-THF to a metabolically active form, which it achieves by catalyzing irreversible, ATP-dependent conversion to 5,10-CH₂-THF (2, 5). This enzyme is also something of an enigma. For one thing, despite the inhibitory effects of its substrate, 5-FCL is not essential in yeast: 5-FCL disruptants had 4-fold more 5-CHO-THF but no other new phenotype (2). For another, phylogenomic profiling (17, 18) indicates that some bacteria lack 5-FCL even though they have SHMT. Lastly, 5-FCL overexpression in human cells lowered the folate level and raised folate turnover rate, suggesting that 5-FCL may have a second function as a folate-degrading enzyme (19).

Another intriguing feature of 5-FCL is that its subcellular location differs among eukaryotes. Whereas the enzyme is largely if not solely cytosolic in yeast and mammals (2, 16, 20, 21), it is exclusively mitochondrial in plants (4). Taken with the key role of mitochondrial SHMT in photorespiration, with the inhibition of SHMT by 5-CHO-THF, and with the presence of 5-CHO-THF in leaf mitochondria, the location of plant 5-FCL

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¶ This abbreviation is used for: 5-CHO-THF, 5-formyltetrahydrofolate; 10-CHO-THF, 10-formyltetrahydrofolate; 5,10-CH₂-THF, 5,10-methylene-tetrahydrofolate; 5-CH₃-THF, 5-methyltetrahydrofolate; 5-FCL, 5-formyltetrahydrofolate cycloligase; SHMT, serine hydroxymethyltransferase; GC-MS, gas chromatography-mass spectrometry; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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FIG. 1. Photosynthetic metabolism and folate interconversions in plant mitochondria. In photosynthesis, glycine coming from photoperoxidase is converted to serine in the mitochondria by the concerted action of the glycine decarboxylase complex (GDC) and SHMT. SHMT also mediates formation of 5-CHO-THF from 5,10-CH=THF; the reverse reaction is catalyzed by 5-PCL, which is solely mitochondrial in plants (4). Possible SHMT inhibition by 5-CHO-THF is shown by a dashed line. Plant mitochondria also contain an isoform of the bifunctional 5,10-methylene-THF dehydrogenase/5,10-CH=H11005 (Leu 11005) (4). There are also extramitochondrial isoforms of both enzymes (15).

implies (i) that this enzyme governs mitochondrial 5-CHO-THF levels and (ii) that, via its effect on 5-CHO-THF levels, 5-PCL could regulate the in vivo activities of SHMT and other folate-linked metabolic reactions in plants that are substantially tolerant to 5-CHO-THF.

EXPERIMENTAL PROCEDURES

Chemical and Reagents—Folates were from Schircks Laboratories (Jona, Switzerland). 10-Formyltetrahydrofolate was prepared from (6R,8S)-5-CHO-THF as described (22). [1-14C]formate (3000 Ci mmol–1) was from PerkinElmer Life Sciences.

Plants and Growth Conditions—Arabidopsis thaliana plants were grown at 22–28 °C in 12-h days (photosynthetic photon flux density 50 μE m–2 s–1) in potting soil irrigated with water. Material was lyophilized to determine dry weight. In experiments at various CO2 levels, plants were grown for 4 weeks in ambient air (~370 μmol of CO2 mol–1), then either kept in ambient air or transferred to high CO2 (300 ± 40 μmol mol–1) or low CO2 (30 ± 20 μmol mol–1) conditions for 5 days. CO2 levels were monitored with a Vernier CO2 sensor (Vernier Software and Technology, Beaverton, OR).

Arabidopsis Mutant—A 5-PCL mutant (28D07) was identified in the Syngenta T-DNA insertion collection (ecotype Columbia) (23). Segregants, wild type or homozygous for the mutation, were identified by PCR using gene-specific primers located 5′ or 3′ of the T-DNA insertion (5′-CTGAACTGTGGAATCATAA-3′ and 5′-GTCTACTTCTTCTC-3′; respectively) and the T-DNA-specific primer 5′-GACTCTGAATTTACAACAATC-3′. DNA was extracted by the “Shorty” protocol available on the website of the University of Wisconsin Biotechnology Center. The insertion site was confirmed by sequencing.

Gel Blot Analyses—Total RNA was extracted from 0.1-g samples of root tissue using RNaseasy kits (Qiagen, Valencia, CA) and treated with DNase (DNA-free™ kit, Ambion, Austin, TX). RNA samples were separated by formaldehyde–1.5% agarose gel (25 µl aliquot) and blotted to Protran® nitrocellulose membrane (Schleicher and Schuell, Keene, NH), hybridized for 4 h at 65 °C in 6× SSC, 0.5% SDS, 5× Denhardt’s solution, and 100 µg ml–1 sonicated salmon sperm DNA, and washed in 0.1× SSC, 0.5% SDS at 65 °C. The 5-PCL probe was a 196-bp ND1-Xhol fragment of pET28b-4T5PCL (4) corresponding to the 3′-half of the 5-PCL open reading frame (Fig. 2A). The RNA probe was a 0.9-kb Smal fragment of a Zamia pumila clone (24). Genomic DNA was isolated as described (25) from 2 g of leaves pooled from 20 plants, digested, separated by 0.8% agarose gel (5 µl aliquot), and blotted to Protran® membrane. Blots were hybridized as above and washed in 0.1× SSC, 0.5% SDS at 37 °C. The probe was a 436-bp fragment of the bar gene amplified using the primers 5′-CATCGTCCACATTACGCTC-3′ (forward) and 5′-GAAGTAGCACCACCGCAAGA-3′ (reverse), and labeled with [α-32P]dCTP by the random primer method. Hybridization was detected by autoradiography.

Folate Analysis—Folates were extracted from leaf tissue (0.5 g) by Polytron homogenization in 10 ml of 50 mM Na-HEPES, 50 mM CHES, adjusted to pH 7.9 with HCl, containing 2% (w/v) sodium ascorbate, and 10 mM β-mercaptoethanol (buffer 1), followed by boiling for 10 min and then centrifuging (13,000 × g, 10 min). The pellet was reextracted the same way, and the extracts were combined. Mitochondria were extracted once with 5 ml of buffer 1. Extracts were treated with 1 ml (leaves) or 0.5 ml (mitochondria) of dialyzed rat plasma at 37 °C for 2 h to deglutamylate folates. Samples were then boiled for 15 min, centrifuged, filtered, and applied to folate affinity columns prepared as described (26). Column volume was reduced to 1 ml for mitochondria. After washing columns with 5 ml (2.5 ml for mitochondria) of 25 mM potassium phosphate, pH 7.0, plus 1% sodium ascorbate (buffer 2) containing 1 mM NaCl, then with 5 ml (2.5 ml for mitochondria) of buffer 2 alone, they were eluted with 5 ml (1 ml for mitochondria) of HPLC mobile phase A (see below) containing 1% ascorbic acid. Elute samples (400 µl) were analyzed by HPLC (27) using a Prodigy 5-μm ODS2 column (3.2 mm, Phenomenex, Torrance, CA) and a four-channel electrochemical detector (CoularArray Model 5600A, ESA, Chelmsford, MA) with potentials set at 0, 300, 500, and 600 mV. The mobile phase was a binary mixture of 28 mM K2HPO4 and 0.59 mM H3PO4, pH 2.5 (A) and a mixture of 75% (v/v) A and 25% CH3CN (B) with a 55-min nonlinear elution program from 90% A to 100% B at 1 ml min–1. Detector response was calibrated with authentic tetrahydrofolate (THF), 5-methyl-THF (5-CH3-THF), 5,10-CH=THF, 5,10-CHO-THF, 10-formyl-THF, and folate acid.

Amino Acid Analysis—Leaf tissue (~160 mg) was frozen in liquid N2, lyophilized, weighed, and pulverized. The resulting powder was extracted by shaking with 0.5 ml each of water and CHCl3 and was then stored at ~20 °C for 24 h before centrifugation. Ribitol and γ-aminobutyric acid were added as internal standards. For HPLC, 20 µl of the aliquot (100 mg of leaves) was derivatized with AccQFluor® reagent (28) (quinoxyN-hydroxysuccinimidylcarbamate; Waters, Milford, MA) in a final volume of 100 µl, and a 20-µl aliquot was analyzed by HPLC-fluorescence according to Waters’ recommendations. For GC-MS, aqueous phase aliquots equivalent to 0.625 mg dry weight were dried under N2 and methoximated and trimethylsilylated in pyridine (final volume 50 µl) as described (28). One µl of the derivatized mixture was injected (pulsed splitless injection, Agilent 6890 series autoinjector, Agilent Technologies, Palo Alto, CA) onto a 60-m DB-5MS column (J&W Scientific, Palo Alto, CA). GC-MS analysis was performed with an Agilent 6890 gas chromatograph and a 5973 series Agilent quadrupole mass spectrometer as described (28).

Isolation of Mitochondria and SHMT Assay—Mitochondria were prepared from leaves of 4-week-old Arabidopsis plants as described (29), with the following modifications. The pellet containing chloroplasts and mitochondria was suspended in 2 ml of a solution containing 20 mM TES-NaOH, pH 7.2, 0.25 mM sucrose, 1 mM EDTA, 2 mM MgCl2, 0.1% bovine serum albumin, 14 mM β-mercaptoethanol, applied to a step gradient composed of 2.5 ml of 21%, 5 ml of 26%, and 3 ml of 47% (v/v) Percoll, and centrifuged at 65,000 × g for 45 min in a swinging bucket rotor. Mitochondria were recovered from the 26–47% Percoll interface, diluted 12-fold in 10 mM Tricine-NaOH, pH 8.0, 1 mM EDTA, 14 mM β-mercaptoethanol, and 0.25 mM sucrose, and then centrifuged at 12,500 × g for 20 min. This step was repeated twice. The final mitochondrial pellet was suspended in 300 µl of 10 mM Tricine-NaOH, pH 8.0, 1 mM EDTA and stored under N2 at ~80 °C until analysis. SHMT was assayed in mitochondrial extracts as described (4). 5-CHO-THF Feeding—Three wild type and three mutant plants (5 weeks old) were washed free of soil, and their root systems were severed beneath water, leaving ~0.5 cm of the main root. The plants were placed in Petri dishes containing 0 or 10 mM 5-CHO-THF in 0.5× Hoagland’s nutrient solution and incubated in light (75 µE m–2 s–1) at 22 °C for 24 h. The plants were then washed, frozen in liquid N2, and lyophilized. Samples (100–150 mg) were taken for GC-MS analysis as above.

RESULTS

Identification and Characterization of an Arabidopsis 5-PCL Mutant—A potential 5-PCL mutant was identified in the Torrey Mesa Research Institute T-DNA mutant collection (23) via the sequence flanking the insert. Resequencing of this region

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confirmed the presence of an insert close to the 3’ end of the sixth intron, which is located within the protein-coding part of the gene (Fig. 2A). Plants homozygous for the mutation and their wild type siblings were identified by PCR and subjected to Southern analysis using a T-DNA sequence (a fragment of the bar gene) as probe (Fig. 2B). Only the mutant plants gave hybridizing bands, establishing that the T-DNA is inserted only at the 5-FCL locus. The multiple banding pattern in Fig. 2B indicates that several concatenated T-DNA copies are present at this locus. Northern analysis of leaf RNA showed no detectable 5-FCL transcript in the mutant (Fig. 2C), indicating a knock-out mutation. The homozygous mutants and their wild type siblings were therefore propagated for further work. When grown in soil at ambient levels of CO₂ (~370 μmol of CO₂ mol⁻¹), visible differences between mutant plants and their wild type siblings were modest. The growth rate of mutant plants was ~20% lower (Fig. 2D), and they showed a flowering delay of about 1 week (Fig. 2E). There was no difference in leaf color or form (Fig. 2E).

**Folate Metabolic Profiling and Its Interpretation**—Folates were treated with conjugase to convert them to monoglutamyl form, purified by affinity chromatography (26), and then separated by HPLC with electrochemical detection (27), which was chosen for its selectivity and sensitivity. These procedures do not distinguish between 10-CHO-THF and 5,10-CH₂-THF, which are interconverted during sample processing and finally both measured as 5,10-CH=THF (27). Efforts were made to preserve 5,10-CH=THF by extraction in maleate buffer (30) and to estimate it as an increase in 5-CH₃-THF following NaBH₄ reduction (31), but this maneuver gave poor results with plant samples. Other HPLC-based procedures likewise fail to distinguish 10-CHO-THF from 5,10-CH=THF, measuring both as 10-CHO-THF (30). It is thus currently not feasible to determine the individual amounts of 10-CHO-THF and 5,10-CH=THF present in vivo, but only their sum (henceforth termed 10-CHO/5,10-CH=THF). Similarly, it should be noted that 5,10-methylene-THF (5,10-CH₂-THF) dissociates completely to THF during processing so that THF measurements are the sum of THF and 5,10-CH₂-THF (henceforth termed THF/5,10-CH₂-THF).

**Folates in Leaves Exposed to Various CO₂ Concentrations**—Plants grown for 4 weeks in ambient air were transferred for 5 days to air containing 30 or 3200 μmol of CO₂ mol⁻¹ or kept in ambient air. The lower CO₂ concentration, which is beneath the CO₂ compensation point, stimulates photorespiration (and hence the glycine → serine flux rate in mitochondria) whereas the higher one suppresses it (32). CO₂ concentration had rather little effect on folate profiles so that the divergences between wild type and mutant leaves were generally similar in all three atmospheres (Fig. 3). As might be expected, 5-CHO-THF levels were higher in the mutant (2.1- to 2.6-fold, significant at p < 0.05). Less expectedly, 10-CHO/5,10-CH=THF levels were also much higher in mutant leaves exposed to 370 and 30 μmol of CO₂ mol⁻¹. Added together, these differences made the total folate content of the mutant significantly higher at the two lower CO₂ levels.

**Mitochondrial Folates**—Because mitochondria in photorespiring leaves are expected to be the main site of 5-CHO-THF formation and the only site of its removal by 5-FCL (Fig. 1), we investigated the impact of the 5-FCL mutation on mitochondrial folate levels of plants grown in ambient air. Four separate mitochondrial preparations were made from wild type or mutant plants (Fig. 4). Despite some variability among the preparations, mitochondria from the mutant clearly showed massive 5-CHO-THF accumulation relative to wild type (on average 8-fold, significant at p < 0.01) and a 120% increase in total folate. The mitochondrial 5-CHO-THF content rose from a mean value of 15% of total mitochondrial folate in the wild type to a mean of 72% in the mutant. The mitochondrial contents of 5-CH₃-THF and 10-CHO/5,10-CH=THF did not change significantly in the mutant, nor did that of 10-formyltetrahydrofolate (which forms readily from 10-CHO-THF in isolated mitochondria).
and mutant mitochondria were 340 to check for a possible compensatory increase in the mutant. No normal growth, led us to measure mitochondrial SHMT activities for 5 days to 30 or 3200 μmol of CO₂ mol⁻¹ to the wild type (3.4-fold at 370 μmol of CO₂ mol⁻¹ and 1.8-fold at 30 μmol mol⁻¹, both significant at p < 0.01).

Besides serine, amino acids such as glutamate and alanine can act as amino donors in the formation of glycine from glyoxylate in the photosynthetic pathway (34). The levels of glutamate, alanine, and also aspartate were substantially reduced in the mutant compared with wild type at 30 μmol of CO₂ mol⁻¹ (Table I) but not at the other CO₂ concentrations (not shown).

**Effect of Supplied 5-CHO-THF on Glycine and Serine Content**—Because exogenous 5-CHO-THF is taken up by Arabidopsis and enters mitochondria (35), we examined the effect of feeding 5-CHO-THF via the transpiration stream to illuminated wild type and mutant plants. Leaf glycine and serine levels were measured after 24 h of continuous light (Fig. 6). Consistent with in vivo inhibition of SHMT, 5-CHO-THF feeding raised glycine levels in wild type and mutant plants significantly (p < 0.05). The increase in glycine was larger in the mutant (9.4 versus 2.5 μmol g⁻¹ dry weight).

**DISCUSSION**

Our data demonstrate that 5-CHO-THF can inhibit the activity of mitochondrial SHMT in vivo and thus provide support for the view that 5-CHO-THF regulates one-carbon metabolism (5). Despite its important implications, there is rather little evidence for or against this view, and it remains controversial (8). However the most striking aspect of our findings is that 5-FCL ablation and the ensuing 5-CHO-THF buildup in mitochondria had so little impact on plant performance. A priori, this impact seemed likely to be devastating, above all to photosynthesizing leaves (4). We therefore conclude that plants are surprisingly tolerant of 5-CHO-THF, as presaged by earlier reports of remarkably high levels of this folate in leaf mitochondria (10, 11). Several mechanisms could contribute to this tolerance.

The first may be the ability of an expanded glycine pool to offset inhibition of SHMT by 5-CHO-THF. Assuming a matrix volume/protein ratio of 1–2 μl mg⁻¹ (36), the concentration of 5-CHO-THF in leaf mitochondria can be estimated from the data in Fig. 4 to be 0.25–0.5 mM in the wild type, and 2–4
Plants were supplied with water or 10 mM 5-CHO-THF via leaves fed with 5-CHO-THF. The shoots of wild type and mutant 0.1 mM significantly reduce mitochondrial SHMT activity insensitivity of mitochondrial enzymes to this compound or mechanism of plant tolerance to 5-CHO-THF is either relative D-riboside transformylase.

Data are means of three replicates ± S.E. Derivatized samples were subjected to GC-MS amino acid analysis. Purine synthesis enzyme aminoimidazole-4-carboxamide-1-clohydrolase, 5,10-methylene-THF dehydrogenase, and the SHMT and glycine decarboxylase alone constitute up to 40% of mitochondrial folate-dependent enzymes: SHMT and glycine decarboxylase alone constitute up to 40% of mitochondrial folate enzymes (45). At such high abundances, it can be calculated from the data of Fig. 4 that even in mutant mitochondria, containing ~4 nmol of 5-CHO-THF mg\(^{-1}\) of protein, folate binding sites probably still outnumber 5-CHO-THF molecules. Furthermore, binding of 5-CHO-THF to SHMT and glycine decarboxylase may decrease the inhibition of other folate-dependent enzymes in mitochondria.

Because 5-CHO-THF is quite chemically stable and 5-FCL is the only enzyme known to metabolize it, the action of SHMT in a 5-FCL mutant would in the long run be expected to convert much of the cellular folate pool to 5-CHO-THF, especially under photorespiratory conditions. That the total leaf 5-CHO-THF pool increased no more than 2.6-fold (to a maximum of 31% of total folate) suggests that there may be another, unknown, way to metabolize 5-CHO-THF, or in effect to detoxify it, and that this contributes to tolerance. The same may hold true of yeast, where the 5-CHO-THF level increased only 4-fold in a 5-FCL knock-out (2), and of those bacteria that apparently lack 5-FCL genes (17, 18). Unlike the 5-FCL reaction, which salvages 5-CHO-THF as an intact folate, any alternative detoxification route seems likely to entail cleavage of the p-aminobenzoate-glutamate bond or the pteridine-p-aminobenzoate bond (2, 46). In support of the latter possibility, exploratory work in our laboratory has shown a large (~15-fold) accumulation of a novel pteridine in the leaves of 5-FCL mutant plants.

Finally, we saw no evidence that 5-FCL moonlights as a folate-degrading enzyme in plants, as has been suggested for the mammalian enzyme (19). Although total folate content indeed increased in the 5-FCL mutant, it did so only under photorespiratory conditions, suggesting that the increase was not due to the mutation per se but rather to its metabolic sequelae.

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**TABLE 1**

| Amino acid | Wild type | Mutant |
|------------|-----------|--------|
| Alanine | 0.80 ± 0.26 | 0.32 ± 0.03 |
| Glutamate | 2.55 ± 0.12 | 1.23 ± 0.10* |
| Aspartate | 1.15 ± 0.07 | 0.22 ± 0.04* |

* Differences between mutant and wild type that are significant at p < 0.05.
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