Folate synthesis in plants: The first step of the pterin branch is mediated by a unique bimodular GTP cyclohydrolase I

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GTP cyclohydrolase I (GCHI) mediates the first and committing step of the pterin branch of the folate-synthesis pathway. In microorganisms and mammals, GCHI is a homodecamer of ~26-kDa subunits. Genomic approaches identified tomato and Arabidopsis cDNAs specifying ~50-kDa proteins containing two GCHI-like domains in tandem and indicated that such bimodular polypeptides occur in other plants. Neither domain of these proteins has a full set of the residues involved in substrate binding and catalysis in other GCHIs. The tomato and Arabidopsis cDNAs nevertheless encode functional enzymes, as shown by complementation of a yeast fol2 mutant and by assaying GCHI activity in extracts of complemented yeast cells. Neither domain expressed separately had GCHI activity. Recombinant tomato GCHI formed dihydroneopterin triphosphate as reaction product, as do other GCHIs, but unlike these enzymes it did not show cooperative behavior and was inhibited by its substrate. Denaturing gel electrophoresis verified that the bimodular GCHI polypeptide is not cleaved in vivo into its component domains, and size-exclusion chromatography indicated that the active enzyme is a dimer. The deduced tomato and Arabidopsis GCHI polypeptides lack overt targeting sequences and thus are presumably cytosolic, in contrast to other plant folate-synthesis enzymes, which are mitochondrial proteins with typical signal peptides. GCHI mRNA and protein are strongly expressed unripe presumably cytosolic, in contrast to other plant folate-synthesis enzymes, and catalytic activity is a dimer. The deduced tomato and Arabidopsis GCHI polypeptides lack overt targeting sequences and thus are presumably cytosolic, in contrast to other plant folate-synthesis enzymes, which are mitochondrial proteins with typical signal peptides. GCHI mRNA and protein are strongly expressed in unripe yeast cells. Neither domain expressed separately had GCHI activity. 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of ~1.5. Cells were twice washed in water and once in 50 mM Tris-HCl, pH 8.0, frozen in liquid N₂, and stored at ~80°C.

cDNA Cloning and Expression. PCR was made with Pfu Turbo (Stratagene) or DyNAzyme EXT (Finnzymes, Helsinki). Constructs were verified by sequencing. Expressed sequence tag AW738516 [encoding Lycopersicon esculentum (Le)GCHI] was obtained from CUGI (Clemson, SC). An Arabidopsis cDNA encoding Arabidopsis thaliana (At)GCHI was obtained by leaf mRNA by using SuperScriptII reverse transcriptase (Invitrogen) and amplified with the primers 5'-ATAATAGAAATCTTGATCAATTAAACTATGGGCCGATTAGTGAGGATGTTTG-3' and 5'-TATTATTATAGCTTTTCAAAAATGAGGACTTGCTGCTTC-3'. The amplification was digested with EcoRI and BglII and cloned into pNCO113 (16). The vector for yeast expression was pVT103-U (17). The LeGCHI coding sequence was amplified by using primers 5'-ACGGGGATCCCTATGGGCCGATTAGTGA3' and 5'-CTGGTATCTTCTGCACAGACC-3'; the separate domains were amplified by using these plus the internal primers 5'-CTGTTCATCTTCTGCACAGACC-3' or 5'-ACGGGGATCCCTATGGGCCGATTAGTGA3' and cloned into pVT103-U (17). The LeGCHI coding sequence was amplified by using primers 5'-GATCATTCACTAGCTCCAGAA-3' and 5'-ATCTTTCCATTTTTACG-3' to give pVT103-U-N, which was transformed into E. coli XL-1. The 1,380-bp Sall–PstI fragment of the pNCO113 construct was cloned into pVT103-U-N, and the resulting plasmid was transformed into XL-1 cells. Yeast transformants were isolated by using the YEASTMAKER system (CLONTECH). For reverse transcription, total RNA was isolated from tomato leaves by using TRIzol reagent (Invitrogen). The RNA was treated with RNase-free DNase (Qiagen); antibodies against the purified protein were raised in rabbits. SDS/PAGE and immunoblotting were as described (19); antiserum was diluted 1:1,000.

GCHI Assays. GCHI activity was assayed by a procedure in which the reaction product, dihydronopterin triphosphate, is oxidized and dephosphorylated to yield neopterin for HPLC analysis (20). Standard assays (40–200 μl) contained 50 mM Tris-HCl, pH 8.0, 0.1 M KCl, 1 mM GTP, and enzyme and were run at 37°C for 1 h. Reactions were stopped on ice with oxidizing solution (0.5% L/1% KI in 1 M HCl, 10 μl per 100-μl reaction) and incubated for 1 h at 23°C before alkaline phosphatase treatment and deproteinization. [Tests showed that the high pH of the phosphatase step converts dihydronopterin triphosphate to the phosphatase-resistant cyclic monophosphate (21, 22). Unless the triphosphate is hydrolyzed during the assay, as occurs by phosphatase action in yeast extracts, the cyclic form is therefore the major product. Chromatography was a modification of the method of Lee et al. (23). Briefly, samples were injected onto an Ultramex C18 (ion pair) column (5 μm, 250 × 4.6 mm, Phenomenex, Belmont, CA) and eluted isocratically with 10 mM Na2HPO₄, pH 6.0, at 1.2 ml/min. Peaks were detected at 350-nm excitation, 450-nm emission). The neopterin peak was quantified relative to d-neopterin standards. Data were corrected for the recovery of 7,8-dihydro-di-n-neopterin-3'-monophosphate spikes added to assays before incubation; recovery rates were ~50%. Pettin standards were from Schircks Laboratories. Dihydronopterin triphosphate was prepared by recombinant E. coli FolE (12), oxidized, and converted to the cyclic monophosphate at pH ~ 10 (21).

Folate Analysis. Pilot studies using deconjugated samples (ref. 24, with modifications) showed that >90% of fruit folate was present as 5-methyltetrahydrofolate. However, because deconjugation can be inhibited by organic ions, the assay was adapted to measure 5-methyltetrahydrofolate polyglutamates. Folate was purified from 1.0–1.5 g of fruit (24) and analyzed by using reverse-phase fluorometric HPLC (25). Authentic 5-methyltetrahydrofolate monoglutamate (Eprova, Saffhausen, Switzerland) was used as a quantitative standard, and folate extracts from human erythrocytes were used to identify the retention times of the 5-methyltetrahydrofolate polyglutamates.

Northern Analysis. Total RNA was isolated from fruit and leaf samples (0.5–1.0 g) as described (26), separated on formaldehyde gels containing 1% agarose, and blotted to Nitrotype membrane (Osmonics, Minnetonka, MN). Blots were hybridized overnight at 65°C in 5× SSPE/5× Denhardt’s solution/0.5% SDS/100 μg/ml fragmented salmon sperm DNA and washed at 37°C in 0.1× SSPE containing 1% SDS (standard saline phosphate/EDTA: 0.75 M NaCl/0.05 M phosphate, pH 7.4/5 mM EDTA). Blots were probed with the PCR-amplified coding sequence of LeGCHI and then stripped and reprobed with an 18S rRNA sequence (27). Probes were labeled with 32P by random priming. Radioactive LeGCHI mRNA bands were detected by autoradiography for 10 days.
Results

Higher Plant Homologs of GCHI Are Bimodular. BLAST searches of GenBank and TIGR databases using the protein sequences of *E. coli* or mammalian GCHIs detected GCHI-like proteins encoded by an *Arabidopsis* gene (At3g07270) and by expressed sequence tag contigs from tomato (TC91226) and *Medicago truncatula* (TC36937). Remarkably, all three predicted proteins have two tandem GCHI domains that share more identity with various eukaryotic GCHIs than with each other (Fig. 2A). To confirm that such bimodular GCHI homologs actually exist in mRNA populations, we sequenced full-length *Arabidopsis* and tomato cDNAs. These cDNAs indeed coded for bimodular proteins (Fig. 2B). Alignments of the domains of the *Arabidopsis* and tomato proteins (henceforth, AtGCHI and LeGCHI) with the well characterized *E. coli* and human GCHIs revealed a second exceptional feature: neither domain has a complete set of the residues deemed essential for substrate binding and catalysis (11, 12), although all are present in the protein as a whole (Fig. 2B). The deduced *M. truncatula* protein shares these heterodox features (not shown). Furthermore, both domains of the plant enzymes lack an EF-hand-like calcium-binding motif that is conserved in all other eukaryotic GCHIs (28) and in fact have small insertions in the motif region (Fig. 2B).

*AtGCHI* and *LeGCHI* Are Functional Enzymes but Their Separate Domains Are Not. To find whether the plant proteins have GCHI activity, their entire coding sequences were cloned into the yeast expression vector pVT103-U and introduced into yeast strain 971/6a. This strain is a fol2 deletant that lacks GCHI activity and is auxotrophic for folate (supplied as 5-formyltetrahydrofolate; ref. 13). Expression of the full-length plant proteins restored the ability to grow without folate (Fig. 3A). No complementation was seen with vector alone (Fig. 3A), and retransformation of 971/6a with rescued plasmid restored folate prototrophy, confirming that the complementation is caused by the encoded plant protein (not shown).

GCHI activity in extracts of complemented 971/6a cells was assayed by a standard HPLC-fluorescence method in which the dihydroneopterin triphosphate reaction product is measured as its oxidized and dephosphorylated derivative, neopterin (20). Activity was detected readily in complemented cells at levels higher than in wild-type yeast but was not found in empty-vector controls (Fig. 3B). The assay product cochromatographed with authentic neopterin (Fig. 3D). To confirm that the direct product of plant GCHI is a triphosphate, the recombinant tomato enzyme was subjected to HPLC to remove endogenous phosphatases (see below), and phosphatase treatment was omitted from the sample work-up; the oxidation step was retained. As expected, this procedure yielded a reaction product that cochromatographed with neopterin triphosphate (Fig. 3E). Further confirmation that this product is the triphosphate was obtained by exposing it to alkaline conditions (pH 10), which gave a product that cochromatographed with cyclic neopterin monophosphate (not shown). This cyclization reaction is diagnostic for the triphosphate (21).
To test whether either of the domains can function on its own, those of LeGCHI were expressed separately in yeast strain 971/6a. The two domains were as shown in Fig. 2B except for the addition of a methionine at the start of the C-terminal domain. Neither domain conferred folate-independent growth (not shown) or produced detectable GCHI activity in cell extracts (Fig. 3B).

**LeGCHI Does Not Show Cooperative Behavior and Is Inhibited by GTP.** Because mammalian and bacterial GCHIs show positive and negative substrate cooperativity, respectively (29, 30), we investigated the kinetic properties of recombinant LeGCHI. The GTP substrate used was chromatographically homogeneous by HPLC. Plots of initial velocity vs. GTP concentration showed no sign of cooperative behavior (which other GCHIs manifest within the range of GTP concentrations we used) but exhibited marked substrate inhibition (Fig. 3C). The apparent $K_m$ and $K_i$ values for GTP were calculated as described by Cleland (31) to be 46 and 173 $\mu$M, respectively. The $K_m$ value is close to the GTP concentration that gives half-maximal velocity ($K_{0.5}$) for the rat enzyme (29) and 50-fold higher than the $K_{0.5}$ for E. coli GCHI (30). Substrate inhibition has not been reported for other GCHIs.

**The LeGCHI Polypeptide Remains Intact in Vivo and Forms Active Dimers.** To check that plant GCHIs are not posttranslationally cleaved into their constituent domains, for which there is precedent in other proteins (32), antibodies raised against LeGCHI were used to probe immunoblots of recombinant LeCGHI from yeast extracts (Fig. 4A). The only band detected had a molecular mass of 91 kDa, which is very close to the predicted value of 50 kDa. This result confirms that the bimodular polypeptide exists as such in vivo.

Because other GCHIs are decamers (11, 12), we investigated the native molecular mass of the plant enzyme. Size-exclusion HPLC showed that recombinant LeGCHI behaved as a single species with an apparent mass of 91 ± 5 kDa (Fig. 4B). The same value was obtained when the substrate GTP (0.1 mM) was added to the column buffer (not shown). These data indicate that the native enzyme is a dimer of 50-kDa subunits.

**LeGCHI Is Expressed Strongly in Unripe but Not Ripe Fruit.** Because nothing is known about expression of the folate-synthesis pathway in fruits, GCHI transcript and protein levels were analyzed in tomatoes harvested at various developmental stages. Leaves were included as a benchmark, because they are known to have
it might seem that the dimer has merely become a covalently bonded unit in plants; but this is not so, because neither of the domains of the plant protein has all the residues identified in other GCHIs as essential for substrate binding and catalysis, and neither domain alone is enzymatically competent. Also, the bimodular plant protein dimerizes to form the active enzyme, and this quaternary structure precludes formation of the active site in the same way as in E. coli and mammalian GCHIs. Table 1 summarizes how the active site residues in these nonplant GCHIs come from three different subunits and shows for the plant enzymes how residues missing from one domain are present in the other such that the protein as a whole has a full set. The two cysteines and one histidine residues that coordinate the catalytically essential zinc ion (Table 1, bold italics) are a special case. Whereas all three are in canonical positions in the plant GCHI C-terminal domain, Cys-181 and His-113 have switched places in the N-terminal domain. Both domains of plant GCHIs thus may be able to bind zinc. Because plant GCHIs seem to violate principles upon which other GCHIs are constructed, determining their molecular structure will be of much interest from the standpoint of reaction mechanism.

In mammals, feedback inhibition of GCHI by the end product tetrahydrobiopterin is mediated by a pantameric GCHI feed-back-regulatory protein (GFRP) that binds specifically to each of the two faces of the toroid-shaped GCHI decamer (35, 36). That plant GCHI has primary and quaternary structures so unlike those of mammals makes it improbable that the plant enzyme has the same type of feedback-inhibitory mechanism. Consistent with this, there are no obvious GFRP homologs in plant genome or expressed sequence tag databases. Moreover, plants do not produce tetrahydrobiopterin. Were mammalian GCHI expressed in plants, it therefore might be deregulated through lack of feedback control, which could provide a way to engineer increased flux to pterins and folate.

The last five steps in folate synthesis, from pterin activation onward, are all mitochondrial in plants, four of them exclusively so, and the corresponding enzymes have obvious transit peptides (5, 6). The lack of a transit sequence in GCHI thus makes it an

active folate synthesis (7, 14). We also measured the fruit levels of 5-methyltetrahydrofolate, which was shown to be >90% of total fruit folate. GCHI mRNA level was highest in unripe (mature green and breaker stage) fruit, when it was near that in leaves (Fig. 5A). As ripening advanced, the mRNA level fell sharply and was barely detectable when the fruit reached full color and was softening (red-ripe stage). GCHI protein likewise was abundant only in unripe fruit and disappeared even faster than the transcript (Fig. 5B). Levels of 5-methyltetrahydrofolate showed a significant downward trend ($P = 0.06$) during ripening (Fig. 5C).

**Discussion**

We have identified tomato and *Arabidopsis* cDNAs specifying GCHI, the first and committing enzyme in the pterin branch of the folate-synthesis pathway. This brings the total number of cloned plant folate-synthesis enzymes to six of a probable total of nine (2). The primary structure of plant GCHIs is without precedent in other organisms, comprising dual GCHI-like domains that are more diverged from one another than from other eukaryotic GCHIs. Such divergence implies that bimodular GCHIs are evolutionarily ancient, as do the phylogenetic distances between the species shown to have them—i.e., tomato, *Arabidopsis*, and *M. truncatula* (33).

Because biochemical and crystallographic evidence indicates that the *E. coli* and mammalian GCHI decamers can be viewed as pantemers made up of tightly associated dimers (11, 12, 34), it might seem that the dimer has merely become a covalently

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Table 1. Conservation of active site residues in the domains of LeGCHI and AtGCHI

<table>
<thead>
<tr>
<th>Residue*</th>
<th>Monomer†</th>
<th>N-terminal domain</th>
<th>C-terminal domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg-65</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lys-68</td>
<td>A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cys-110</td>
<td>B</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>His-112</td>
<td>B</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>His-113</td>
<td>B</td>
<td>(Cys)</td>
<td>+</td>
</tr>
<tr>
<td>Ile-132</td>
<td>C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ser-135</td>
<td>C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lys-136</td>
<td>C</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Arg-139</td>
<td>C</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Gln-151</td>
<td>B</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Glu-152</td>
<td>B</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>His-179</td>
<td>B</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cys-181</td>
<td>B (His)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arg-185</td>
<td>B</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

*Numbered by reference to *E. coli* GCHI. Residues involved in zinc binding are in bold italics.
†A, B, and C denote the three different GCHI monomers that contribute the active site residues in the *E. coli* enzyme.
‡Plus (+) indicates conserved or conservatively replaced residues; minus (−) indicates nonconserved residues; +/− indicates conservation in *Arabidopsis* but not tomato. Amino acids that replace His-113 and Cys-181 in the N-terminal domain are in parentheses.
exception and indicates that it is most probably a cytosolic enzyme. If GCHI indeed is cytosolic, it follows that mitochondria must import the pterin moiety of folate from the cytosol. Because genomic data suggest that the PABA moiety of folate is made in chloroplasts (2), coordinated reactions in three subcellular compartments and three membrane transport steps may be needed to produce folates in plants. This arrangement is not found in other eukaryotes, the folate pathway enzymes of which are predominantly cytosolic.

The strong expression of GCHI in unripe fruits implies that fruits synthesize their own supply of pterin—and, by extension, folate—rather than rely on import from leaves. This inference is corroborated by the existence of tomato fruit expressed sequence tags specifying three other folate-synthesis enzymes (e.g., GenBank AW223881, BF050827, and BE433834). The steep decline in GCHI expression after the mature green stage, when folate levels begin to fall, implies a collapse of the fruit’s capacity to maintain its folate-synthesis machinery, and hence to sustain its folate levels in the face of ongoing turnover. The collapse in GCHI expression presumably is programmed, because ripening-related genes are still being induced when it occurs (37). Fruit folate content therefore might be enhanced by prolonging the expression of GCHI and perhaps other folate-synthesis enzymes.

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