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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 proteins 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 imported. As ripening advances, GCHI expression declines sharply, in contrast to other enzyme activities. GCHIs. The tomato and Arabidopsis GCHI cDNAs, and showed that they encode unique bimodular enzymes. Because nothing was known about folate synthesis in fruits, we analyzed GCHI expression and folate levels during fruit development.

Materials and Methods

Plants and Growth Conditions. Tomato (Lycoopersicon esculentum Mill. cv. MicroTom) plants were grown in a greenhouse (maximum temperature, 27°C) or a growth chamber (16-h days, irradiance 200 μmol of photons/m²·s⁻¹, 23°C/8-h nights, 20°C) in potting mix with appropriate fertilizer and pesticide regimes. Fruits and leaves were frozen in liquid N₂ and held at −80°C until use.

Yeast Strains and Culture Conditions. Strains 971/6c (Mata ade2-1 his3-11,15 leu2-3,112 ura3-1 can1) and 971/6a (Mata ade2-1 his3-11,15 leu2-3,112 ura3-1 can1 fol2::HIS3) (13) were obtained from M. L. Agostoni Carbone (Università di Milano, Milan). Yeast was cultured at 30°C in synthetic minimal medium (0.7% Difco yeast nitrogen base without amino acids, 2% glucose) plus appropriate supplements. When needed, 6R,6S)-5-formyltetrahydrofolate (Ca²⁺ salt, Schircks Laboratories, Jona, Switzerland) was added at a final concentration of 50 μg/ml⁻¹. For enzyme extraction, 1-liter cultures were grown to an A600 value

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Abbreviations: PABA, p-aminobenzoate; GCHI, GTP cyclohydrolase I; Le, Lycopersicon esculentum; At, Arabidopsis thaliana.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY069920 and AF489530).

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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 N_2, 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 (Stratagene) or DyNAzyme EXT (Finnzymes, Helsinki). Constructs were ligated between the primers 5'-ATAATAGAATCTTAAAGAAGAGAAATTAACTATAGGCGCATTAAGTGAGGATGTTTGG-3' and 5'-TATTATTAAGCTTCTCCAAATTTGAGCTTCTGTCCT-3'. The amplificate was digested with EcoRI and BglII and cloned into pNC0113 (16). The vector for yeast expression was pVT103-U (17). The LeGCHI coding sequence was amplified by using primers 5'-ACCGGGATCCTAGGGCAGTATTAGATGAA-3' and 5'-CTGTCACTTTCTCGCAGACACC-3'; the separate domains were amplified by using these plus the internal primers 5'-CTGTCACTTTCTAGGCGCATTAAGTGAGGATGTTTGG-3' and 5'-ACCGGGATCCTAGGGCAGTATTAGATGAA-3'. The plasmids were ligated with BamHI and SmaI sites of pVT103-U and introduced into *E. coli* DH10B. For AtGCHI, pVT103-U was digested with BamHI and ScaI and ligated with a synthetic double-stranded oligonucleotide (5'-GTTACATGGCGCGCATTAAGTGAGGATGTTTGG-3'/5'-GGCATTTGACACTCTTCTATGCGCATTAAGTGAGGATGTTTGG-3') to give pVT103-U-N, which was transformed into *E. coli* XL-1. The 1,380-bp ScaI–PstI fragment of the pNC0113 construct was then cloned into pVT103-U-N, and the resulting plasmid was transformed into XL-1 cells. Yeast was transformed by using the YEASTMAKER system (CLONTECH). For plasmid transformation, plasmid DNA was isolated from yeast cells by a phenol-chloroform procedure and amplified in *E. coli* DH10B cells before reintroduction into yeast. To isolate histidine-tagged LeGCHI in *E. coli*, the coding sequence was amplified by using the primers 5'-ACGTGAGCTTCAATAGGGCAGTATTAGAAG-3' and 5'-GATCTCTGGAGCTTCTGCGCAAGAC-3', digested with BamHI and XhoI, cloned into pET-28b (Novagen), and transformed into DH10B cells. This construct was then expressed in BL21 (DE3) cells.

Protein Extraction and Analysis. Operations were at 0–4°C. Pelleted yeast cells from 0.5 liters of culture were suspended in 2 ml of 0.1 M Tris-HCl, pH 8.0, and shaken with 0.5-mm zirconia-silica beads in a MiniBeadBuster (Biospec Products, Bartlesville, OK) at top speed for 6 × 30 s. The extracts were cleared by centrifugation (25,000 × g, 30 min) and desalted on PD-10 columns (Amersham Pharmacia) equilibrated in 50 mM Tris-HCl, pH 8.0, containing 0.1 M KCl and 10% glycerol.

Extracts were routinely frozen in liquid N_2 and stored at −80°C, which preserves GCHI activity. Tomato fruits were ground in 0.1 M Tris-HCl, pH 8.0, containing 5 mM EDTA and 3% polyvinyl-polypyrrolidone and centrifuged (10,000 × g, 15 min) to clear. Native molecular mass was estimated by using a Waters 626 HPLC system equipped with a Superdex 200 HR 10/30 column (Amersham Pharmacia). The elution buffer was 50 mM Tris-HCl, pH 8.0, containing 0.1 M KCl. Protein was estimated by Bradford’s method (18) using BSA as standard. Histidine-tagged LeGCHI from isopropyl β-D-thiogalactoside-induced *E. coli* cultures was isolated under denaturing conditions by using Ni²⁺–nitrilotriacetic acid resin (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, dihydropterin 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 started on ice with oxidizing solution (0.5% I₂/1% KI in 1 M HCl, 10 μl per 100-μl reaction) and incubated for 1 h at 23°C before alkaline phosphate treatment and deproteinization. [Tests showed that the high pH of the phosphate step converts dihydropterin triphosphate to the phosphate-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⁻¹. Peaks were detected by a Jasco FP 920 fluorescence detector (350-nm excitation, 450-nm emission). The neopterin peak was quantified relative to deoxopterin standards. Data were corrected for the recovery of 7,8-dihydro-d-neopterin-3′-monophosphate spikes added to assays before incubation; recoveries were >50%. Pterin standards were from Schircks Laboratories. Dihydropterin 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, 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 (Epova, Schaffhausen, Switzerland) was used as a quantitative standard, and folate extracts from human erythrocytes were used to identify the recycling 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 nylon membranes (Osmonics, Minnetonka, MN). Blots were hybridized overnight at 65°C in 5× SSPE/5× Denhardt’s solution/0.5% SDS/100 μg/ml-¹ 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 deletion 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), and retransformation of 971/6a with rescued plasmid restored folate prototrophy, confirming that the complementation is caused by the encoded plant protein (not shown).

Supporting Information

**Fig. 2.** Primary structures of deduced plant GCHI polypeptides. (A) Scheme showing that the tandem plant GCHI-like domains share 34–42% identity with various eukaryotic GCHIs but only 26–28% identity with each other (arrows and % values). Red bars denote conserved sets of active site residues, and white bars show where these sets are not conserved in plant GCHIs. (B) Amino acid sequence alignment of *E. coli* GCHI (Ec, GenBank accession no. P27511), human GCHI (Hs, CA877392), and the N- and C-terminal domains of tomato (Le, AY069920) and *Arabidopsis* (At, AF489530) GCHIs. Numbering is that of *E. coli* GCHI. Residues 1–30 of human GCHI are omitted. Identical residues are shaded in black, and similar ones are shaded in gray. Dashes are gaps introduced to maximize alignment. Triangles mark the conserved active site residues of the *E. coli* and human enzymes; red color denotes participation in zinc binding (11). Blue circles mark the Ca²⁺-coordinating residues of the EF-hand-like motif conserved in animal and yeast GCHIs (23).
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 μM, respectively. The $K_m$ value is close to the GTP concentration that gives half-maximal velocity ($K_m/2$) for the rat enzyme (29) and 50-fold higher than the $K_m$ 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 LeGCHI from yeast extracts (Fig. 4A). The only band detected had a molecular mass of 51 kDa, which is very close to the predicted value of 50 kDa. This result confirms that the bimodular polypeptide exists in vivo as such.

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...
we were able to identify 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 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 cysteine 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 pentameric GCHI feedback-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
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 BEA43834).

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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