Isolation, Characterization, and Functional Expression of cDNAs Encoding NADH-dependent Methylenetetrahydrofolate Reductase from Higher Plants*

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Methylene tetrahydrofolate reductase (MTHFR) is the least understood enzyme of folate-mediated one-carbon metabolism in plants. Genomics-based approaches were used to identify one maize and two Arabidopsis cDNAs specifying proteins homologous to MTHFRs from other organisms. These cDNAs encode functional MTHFRs, as evidenced by their ability to complement a yeast met12 mutant, and by the presence of MTHFR activity in extracts of complemented yeast cells. Deduced sequence analysis shows that the plant MTHFR polypeptides are of similar size (66 kDa) and domain structure to other eukaryotic MTHFRs, and lack obvious targeting sequences. Southern analyses and genomic evidence indicate that Arabidopsis has two MTHFR genes and that maize has at least two. A carboxyl-terminal polyhistidine tag was added to one Arabidopsis MTHFR, and used to purify the enzyme 640-fold to apparent homogeneity. Size exclusion chromatography and denaturing gel electrophoresis of the recombinant enzyme indicate that it exists as a dimer of ~66-kDa subunits. Unlike mammalian MTHFR, the plant enzymes strongly prefer NADH to NADPH, and are not inhibited by S-adenosylmethionine. An NADH-dependent MTHFR reaction could be reversible in plant cytosol, where the NADH/NAD ratio is 10−3. Consistent with this, leaf tissues metabolized [methyl-14C]methylenetetrahydrofolate to serine, sugars, and starch. A reversible MTHFR reaction would obviate the need for inhibition by S-adenosylmethionine to prevent excessive conversion of methylene- to methylenetetrahydrofolate.

Methylenetetrahydrofolate reductase (MTHFR)1 catalyzes the reduction of 5,10-methylenetetrahydrofolate (CH2-THF) to 5-methyltetrahydrofolate (CH3-THF), which then serves as a methyl donor for methionine synthesis from homocysteine. The MTHFR proteins and genes of Escherichia coli and mammalian liver have been characterized (1–4), and MTHFR genes have been identified in Saccharomyces cerevisiae (5) and other organisms. The MTHFR of E. coli (MetF) is a homotramer of 33-kDa subunits that prefers NADH as reductant (1), whereas mammalian MTHFRs are homodimers of 77-kDa subunits that prefer NADPH and are allosterically inhibited by S-adenosyl-methionine (AdoMet) (2, 3). Two domains have been identified in mammalian MTHFR polypeptides. The NH2-terminal catalytic domain (about 40 kDa) shows 30% sequence identity to E. coli MetF and, like MetF, contains FAD as a noncovalently bound prosthetic group (2). The COOH-terminal domain contains the AdoMet binding site; [methyl-3H]AdoMet photoaffinity labeling located this site about 50 residues from the junction between the domains (2, 3). Yeast and other eukaryotic MTHFRs have a two-domain structure similar to the mammalian enzyme (5, 6).

The MTHFR reaction in liver is physiologically irreversible, due to a combination of the large standard free energy change for the reduction of CH2-THF by NADPH (ΔG°′ = −5.2 kcal mol−1) and the high NADPH/NADP ratio in the cytoplasm (7, 8). A corollary of this irreversibility is that MTHFR has the potential to deplete the pool of CH2-THF, reducing its availability for synthesis of thymidylate and purines (9, 10). The AdoMet sensitivity of the liver enzyme functions to check such depletion, leaving CH2-THF available for other metabolic demands (9, 10). Thus, mammalian MTHFR commits one-carbon units to methyl group synthesis and is considered to have a key regulatory role in one-carbon metabolism.

In contrast to the detailed information about MTHFR from mammals and E. coli, there are few data on plant MTHFR and no genes have been identified (11, 12), making it the least understood enzyme of folate-mediated one-carbon metabolism in plants. MTHFR activity has been detected in crude extracts of pea tissues using a CH3-THF-menadione oxidoreductase (i.e. reverse direction) assay, and found to be insensitive to methionine (13). The reductant has not been identified. No plant MTHFRs have been purified, and the size and number of their subunits remain unknown. This dearth of information on plant MTHFRs and their regulatory properties has become critical

acid; AdoMet, S-adenosyl-l-methionine; PCR, polymerase chain reaction; EST, expressed sequence tag; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography; SMM, l-S-methionine; PPF, photosynthetic photon flux density; NTA, nitritriaicetic acid; E, einstein(s); TLC, thin layer chromatography.

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with the start of work on plant metabolic engineering, because success in many current projects may depend upon understanding and modifying the mechanisms whereby plants balance the demands for methyl groups and other one-carbon moieties. Such projects include engineering the accumulation of betaines or methylated polyols, modifying lignins, and enhancing the synthesis of pharmaceutical alkaloids (14–16).

In this study, we used genomics-based approaches to identify plant MTHFR cDNAs, and expressed them in yeast. The recombinant enzymes were partially characterized, providing a foundation for more detailed study of their catalytic and regulatory properties. We identified cDNAs from plants with the C3 and C4 pathways of photosynthesis (Arabidopsis and maize, respectively) because C2 and C4 species differ in one-carbon metabolism, the former having a large photosynthetic carbon flux through glycine and serine (17). In addition, we developed a sensitive and specific NAD(P)H-CH2-THF oxidoreductase (i.e., forward direction) radioassay that can be used with crude extracts. The results indicate that, in contrast to the mammalian enzymes, the MTHFRs from Arabidopsis and maize use NADH as the reductant, and that AdoMet does not feedback-inhibit their activity.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—[14C]Formaldehyde (53 mCi mmol−1) was purchased from NEN Life Science Products, and (GR6,6S)-methyl-1-[14C]CH2-THF (56 mCi mmol−1) from Amersham Pharmacia Biotech; specific radioactivities were adjusted to the desired values with unlabeled compound. (GR6,6S)-Tetrahydrofolic acid (THF) and (GR6,6S)-CH2-THF were obtained from Schircks Laboratories (Jona, Switzerland). The purity of THF was 86%, estimated by letting the NADH-CH2-THF oxidoreductase reaction go to completion. CH2-THF and 14CH2-THF were dissolved in 8 mM sodium ascorbate and stored at −80 °C. THF was dissolved just before use in N2-gassed 0.25 M triethanolamine-HCl, pH 8.0.

 успех в многочисленных проектах может зависеть от понимания и модификации механизмов, посредством которых растения балансируют свои потребности в метиловых группах и других однометиленовых компонентах. Такие проекты включают инженерию накопления захаров или метиловых полигидратов, модификацию лигнина, и усиление синтеза фармацевтических алкалоидов (14–16).

В этом исследовании мы использовали геномно-ориентированные подходы для идентификации генов MTHFR из растений, и экспрессии их в дрожжах. Рекомбинантные протеины были частично характеризованы, предоставляя фундамент для более детального исследования их каталитических и регуляторных свойств. Мы идентифицировали субъединицы из растений с C3 и C4 путями фотосинтеза (Arabidopsis и кукуруза, соответственно) благодаря тому, что C2 и C4 виды отличаются по однокарбонному метаболизму, первый из которых имеет большую фотосинтетическую углеродную цепь через глицин и серин (17). В дополнение к этому, мы разработали чувствительный и специфический радиоассауил NAD(P)H-CH2-THF оксидоредуктазы (т.е., реакцию в направлении образования CH2-THF) для использования с неразбавленными экстрактами. Результаты свидетельствуют о том, что в отличие от молекулярных эквивалентов, MTHFR из Arabidopsis и кукурузы используют NADH в качестве восстановителя, а AdoMet не ингибирует их активность.

**ПРОЦЕДУРЫ ЭКСПЕРИМЕНТА**

**Химические вещества**—[14C]Фormaldehyde (53 мкCi ммоль−1) был приобретен от NEN Life Science Products, а (GR6,6S)-метил-[14C]CH2-THF (56 мкCi ммоль−1) от Amersham Pharmacia Biotech; радиоактивность была подстроена до заданных значений с помощью неактивированного продукта. (GR6,6S)-Тетрагидрофольная кислота (THF) и (GR6,6S)-CH2-THF были получены от Schircks Laboratories (Jona, Швейцария). Пurity of THF was 86%, оцениваемая по остаточным THF в образцах реакции, которая достигает полного окисления. CH2-THF и 14CH2-THF были заморожены в 8 mM аскорбиновой кислоты и хранились при −80 °C. THF был разведен перед использованием в N2-гassed 0.25 M триэтаноламин-НCl, pH 8.0.

Реакции идентификации генов MTHFR из растений с C3 и C4 путями фотосинтеза (Arabidopsis и кукуруза, соответственно) благодаря тому, что C2 и C4 виды отличаются по однокарбонному метаболизму, первый из которых имеет большую фотосинтетическую углеродную цепь через глицин и серин (17). В дополнение к этому, мы разработали чувствительный и специфический радиоассауил NAD(P)H-CH2-THF оксидоредуктазы (т.е., реакцию в направлении образования CH2-THF) для использования с неразбавленными экстрактами. Результаты свидетельствуют о том, что в отличие от молекулярных эквивалентов, MTHFR из Arabidopsis и кукурузы используют NADH в качестве восстановителя, а AdoMet не ингибирует их активность.
20 min, and stopped by adding 1 ml of 100 mM formaldehyde. After standing for 20 min at 24 °C (to allow $^{14}$C to exchange out of CH$_2$-THF), 0.2 ml of a slurry of AG-50(H$^+$) resin (1:1 with water) was added to bind $^{14}$CH$_3$-THF. The resin was washed with 3 x 1.5 ml of 100 mM formaldehyde, mixed with 1 ml of scintillation fluid, and counted. The counting efficiency was 40%, determined using assays spiked with $^{14}$CH$_3$-THF. The identity of the reaction product was verified by reverse-phase HPLC (27). NADP phosphatase activity was measured by incubating extracts with 10 mM NADP in 100 mM potassium phosphate buffer, pH 7.2, at 30 °C for 30 min, followed by enzymatic assay of NAD using yeast alcohol dehydrogenase.

$methyl-^{14}$C/CH$_3$-THF Metabolism—Arabidopsis rosettes (240 ± 30 mg) or sets of three maize leaf discs (11 mm diameter, 70 ± 3 mg/3 discs, cut from a young blade and scarified with eight radial cuts on the abaxial surface) were allowed to absorb 0.5 µCi (9 nmol) of $[^{14}C]$CH$_3$-THF dissolved in 20 µl of 8 mM sodium ascorbate, minus or plus 25 mM L-serine. Label was fed to rosettes via the severed root, and to discs via the cuts; after uptake, the feeding solution was replaced by water or 25 mM serine. Incubation was in the light (PPFD 5 ± 150 µEm$^2$s$^{-1}$) at 28 °C for 3.5 h. Tissues were extracted with 80% acetone, and the extract was separated into amino acid, organic acid/phosphate ester, and sugar fractions using AG-50(H$^+$) and AG-1 (formate) columns (28). Starch in the insoluble residue was hydrolyzed in 1 M HCl (4 h, 100 °C), and the [14C]glucose formed was purified by ion exchange as above. Amino acids were separated on cellulose TLC plates in n-butanol:acetic acid:water (6:2:2, v/v/v) and by electrophoresis in 0.6 M HCOOH, 1.5 M CH$_3$COOH at 1.8 kV, 4 °C, for 20 min; detection was with ninhydrin. Serine and glycine zones were scraped from electrophoresis plates for $^{14}$C assay. Sugars were separated by TLC on cellulose plates in n-propanol:ethyl acetate:water (7:1:2, v/v/v) and detected with alkaline KMnO$_4$. Samples spiked with $[^{14}C]$CH$_3$-THF were included as controls.

Southern Analyses—Arabidopsis genomic DNA was isolated from leaves as described (29). One-µg samples of the isolated DNA were digested, separated in 0.7% agarose gels, and transferred to supported nitrocellulose membrane (NitroPure, MSI) as described by Sambrook et al. (30). The blots were hybridized overnight at 58 °C in 5 x SSC, 5 x Denhardt's solution, 1% SDS, 1% EDTA, and 100 µg ml$^{-1}$ sonicated salmon sperm DNA, and washed at low stringency (1 x SSC, 0.1% SDS, 37 °C (30)). The probe was the full-length AtMTHFR-1 cDNA. Maize genomic DNA was prepared from 3-day-old seedlings as described (31); 6.5-µg samples were digested, separated in 0.7% agarose gels, and transferred to Duralon-UV membrane (Stratagene). Hybridization was at 42 °C in 6 x SSC, 5 x Denhardt's solution, 0.5% SDS, 50% formamide.

FIG. 1. Alignment of the deduced amino acid sequences of plant MTHFRs with those from human, S. cerevisiae, and E. coli. Identical residues are shaded in black, similar residues in gray. Dashes are gaps introduced to maximize alignment. Asterisks mark residues that interact with the FAD prosthetic group in E. coli (6). The bar indicates the hydrophilic bridge region between the domains (3). The triangle shows the position of an artificial 12-residue insert in the Arabidopsis protein (AAC23420) predicted from genomic sequence. The arrow near the NH$_2$ terminus of the human sequence marks an alternative start site (4). At-1 and At-2, AtMTHFR-1 and -2; Zm-1, ZmMTHFR-1; Hs, human MTHFR (CAB41971); Sc, S. cerevisiae Met13 (P53128); Ec, E. coli MetF (P00394). Because the AtMTHFR-2 cDNA lacked the first six nucleotides of the coding sequence, the first two residues were deduced from the genomic sequence.
and 100 μg ml⁻¹ salmon sperm DNA. Washing was at low stringency (0.1× SSC, 0.1% SDS, 25 °C). The probe was the full-length ZmMTHFR-1 cDNA. Probes were labeled with ³²P by the random primer method. Radioactive bands were detected by autoradiography.

RESULTS

Genomics-based Cloning of MTHFR cDNAs from Arabidopsis and Maize—For Arabidopsis, the strategy was based on a sequence from chromosome II whose conceptual translation product (unknown protein, GenBank accession no. AAC23420) is homologous to eukaryotic MTHFRs. BLAST searches using the deduced cDNA corresponding to AAC23420 detected 15 Arabidopsis ESTs of two types, one essentially identical to the AACTAC420 nucleotide sequence, the other differing by ~15%. Sequencing a nearly full-length representative of each type (both from hypocotyl libraries) confirmed that they encode polypeptides that are 86% identical to each other and ~43% identical to human and yeast MTHFRs (Fig. 1). The deduced proteins are designated AtMTHFR-1 (592 residues, 66.3 kDa) and AtMTHFR-2 (594 residues, 66.8 kDa). AtMTHFR-2 is identical to the AAC23420 conceptual translation product except that the latter has a 12-residue insert (Fig. 1, triangle) attributable to an error made by the gene-prediction algorithm.

For maize, a homology-based PCR strategy was adopted. Two amino acid sequences conserved in eukaryotic MTHFRs were used to design degenerate PCR primers, which amplified a ~1500-base pair fragment from a root cDNA template. Screening a root library with this fragment yielded 10 apparently full-length cDNAs with the same sequence. They encode a 593-residue (66.4 kDa) protein (ZmMTHFR-1) that is 77% identical to AtMTHFR-1 (Fig. 1). Twelve maize MTHFR ESTs were found in GenBank and Pioneer data bases, all encoding ZmMTHFR-1.

Fig. 1 shows that the deduced plant proteins are homologous to human and yeast MTHFRs throughout their entire length, and appear to lack targeting sequences (e.g. chloroplast or mitochondrial transit peptides). In the NH₂-terminal catalytic domain, of the 19 residues shown to interact with the FAD that the latter has a 12-residue insert (Fig. 1, triangle) attributable to an error made by the gene-prediction algorithm. The histidine-tagged AtMTHFR-1 enzyme was purified 640-fold by two cycles of affinity chromatography on nickel-NTA resin (Table I). The specific activity of the purified enzyme was found to be unstable, losing about half its activity during 3 h on ice. To investigate the mass and integrity of MTHFR subunits, the purified protein was analyzed by denaturing gel electrophoresis (Fig. 3). A 64-kDa band was evident, consistent with the size of the deduced polypeptide, and no bands of lower molecular mass. This demonstrates that the plant MTHFR protein isolated from yeast is not cleaved at the junction between the domains, a site that is particularly protease-sensitive in mammalian MTHFR and at which cleavage results in loss of AdoMet inhibition (2, 3). In the purification experiment documented in Table I and Fig. 3, a mixture of protease inhibitors (see “Experimental Procedures”) was added to the buffers. Because very similar results were obtained when PMSF (1 mM) alone was added (results not shown), for all other

Affinity Purification of Histidine-tagged MTHFR and Molec-
The starting material was 1.5 g (wet weight) of cells from a 0.5-liter culture. Proteins were extracted and desalted in buffers containing a proteinase inhibitor mixture (see "Experimental Procedures"). In cycle 1, enzyme was bound at pH 7.5 to nickel-NTA resin, using 50 mM sodium phosphate buffer containing 300 mM NaCl and 40 mM imidazole; the imidazole concentration was raised to 80 mM for washing, and to 400 mM for elution. After diluting the imidazole concentration to 40 mM, the process was repeated for cycle 2 except that elution was with 100 mM imidazole. Activity was measured at 30 °C using the CH3-THF-menadione oxidoreductase assay, with 20% methanol as the solvent for menadione. One millimilliequivalent of AdoMet (nmol of CH2-THF min-1)

### Table I

| Step           | Protein (mg) | Activity (milliunits) | Specific activity (milliunits mg-1) | Yield (%) | Purification (fold) |
|----------------|--------------|-----------------------|-----------------------------------|-----------|--------------------|
| Desalted extract | 21.4         | 230                   | 10.8                              | 100       | 1                  |
| Cycle 1 eluate  | 0.042        | 89.7                  | 2140                              | 39        | 200                |
| Cycle 2 eluate  | 0.0044       | 30.4                  | 6920                              | 13        | 643                |

**Fig. 3. Molecular mass of denatured AtMTHFR-1.** The histidine-tagged form of AtMTHFR-1 was affinity-purified by two cycles of nickel-NTA chromatography, separated by SDS-polyacrylamide gel, and stained with Coomassie blue. Proteins were extracted and desalted in buffers containing a proteinase inhibitor mixture (see "Experimental Procedures"). Lane 1 was loaded with 25 μg of protein from the fraction not bound by the resin, and lane 2 with 0.22 μg of purified protein. The fractions analyzed were from the experiment summarized in Table I. The positions of molecular mass markers (kDa) are indicated.

work we used PMSF. The molecular mass of the native AtMTHFR-1 enzyme was estimated by size exclusion chromatography (results not shown). The protein migrated as a symmetrical peak with an apparent molecular mass of 141 kDa, which is consistent with a dimer of 66-kDa subunits.

**Pyridine Nucleotide Preference—NAD(P)H-CH2-THF oxidoreductase activity cannot be measured spectrophotometrically in crude extracts due to the presence of NAD(P)H oxidase (26), and spectrophotometric assays are in any case fairly insensitive. We therefore developed a radiometric assay to study the pyridine nucleotide specificity of MTHFRs using desalted crude extracts or small amounts of purified enzyme. In this assay, 14CH2-THF (prepared from THF and excess H2[CH2CHO]) is incubated with enzyme, NAD(P)H, and an NAD(P)H recycling system (to prevent any NAD(P) formed from supporting 14CH2-THF oxidation by CH2-THF dehydrogenases). Label remaining in 14CH2-THF is then exchanged out into an excess of unlaabeled HCHO and the 14CH2-THF formed is bound to a cation exchange resin, which is washed and counted. The assay was validated by comparing extracts of RRY3 (MTHFR-deficient) and RRY3 expressing AtMTHFR-1. No activity was detected in RRY3; product formation with the AtMTHFR-1 extract was dependent on pyridine nucleotide and THF, and slightly promoted by FAD (Fig. 4A). The reaction product was confirmed to be 14CH2-THF by reverse-phase HPLC (Fig. 4B).

Using this assay, the three recombinant plant MTHFRs were found to strongly prefer NADH; the activities with 200 μM NADPH were <2% of those with 200 μM NADH, which was a saturating concentration (Table II). Recombinant human enzyme (HsMTHFR) was tested as a control and shown to be NADPH-dependent (Table II), as it is when extracted from liver (2). The NADH-CH2-THF oxidoreductase/CH2-THF-menadione oxidoreductase activity ratio for the plant enzymes was 0.9 ± 0.1, similar to the corresponding ratio for mammalian MTHFR (25, 32).

**Sensitivity to S-Adenosylmethionine and S-Methylmethionine—** Recombinant plant MTHFR activity in desalted extracts was tested for inhibition by high concentrations (1–2 mM) of AdoMet using both NADH-CH2-THF oxidoreductase and CH2-THF-menadione oxidoreductase assays. Extracts were preincubated at 24–30 °C with AdoMet (or buffer for controls) before assays, because onset of AdoMet inhibition is slow (25, 26). Recombinant human enzyme (HsMTHFR) was used as a positive control to check that expression in yeast did not desensitize it to AdoMet. In both assays, the activity of the human enzyme was strongly inhibited by AdoMet, whereas that of ZmMTHFR-1 was unaffected, AtMTHFR-1 was stimulated by 10–20%, and AtMTHFR-2 was stimulated by 50–70% (Tables II and III). The effect of S-methylmethionine (SMM) was also tested, because SMM is a major plant metabolite whose levels can exceed those of AdoMet (33). Physiological concentrations of SMM (2–5 mM) had no effect on either CH2-THF-menadione oxidoreductase (Table III) or NADH-CH2-THF oxidoreductase activities (results not shown). Methionine (5 mM) or S-adenosylhomocysteine (2 mM) were also found to have no effect (results not shown).

**NADH Preference and S-Adenosylmethionine Insensitivity of Purified AtMTHFR-1—** To confirm that the pyridine nucleotide specificity and AdoMet response of the purified recombinant protein are the same as those observed in desalted extracts, the histidine-tagged form of AtMTHFR-1 was tested (Table IV). The instability of the purified enzyme resulted in significant loss of activity during preincubation with AdoMet or buffer alone. The results with purified enzyme nonetheless mirrored those with extracts: the enzyme strongly preferred NADH and was not inhibited by AdoMet; as for crude extracts, there was an apparent stimulation by AdoMet. However, in this case it was shown to be due principally to slower loss of activity during preincubation when AdoMet was present, i.e. to a stabilizing effect of AdoMet.

**S-Adenosylmethionine-insensitive NADH-CH2-THF Oxidoreductase Activity in Plant Extracts—** To rule out the possibility that the NADH-preference and AdoMet-insensitivity of the recombinant plant enzymes are artifacts of the yeast expression system, enzymes extracted from Arabidopsis, maize and two other plants were tested (Table V). In root and leaf extracts of all species, the MTHFR activity showed a strong preference for NADH and was not inhibited by AdoMet; the activities of the extracts were up to 100-fold greater than those in liver. That the ratios of NADPH- to NADH-dependent activities were higher for plant extracts than for recombinant enzymes is attributable to conversion of NADP(H) to NAD(H) by phosphatases in the plant extracts. NADP phosphatase activities...
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TABLE II
Pyridine nucleotide preferences and S-adenosylmethionine sensitivities of NAD(P)/H-CH$_2$-THF oxidoreductase activities in cell-free extracts of transformed yeast

Desalted crude extracts of yeast cells expressing plant or human MTHFRs were assayed for NAD(P)/H-CH$_2$-THF oxidoreductase activity as described under "Experimental Procedures" using NADH or NADPH as reductant, minus or plus 1 mM AdoMet. Extracts were preincubated for 15 min at 24 °C with buffer or AdoMet before the assays. Data are means of 3 replicates ± S.E.

| cDNA expressed | Control | +AdoMet | +SMM |
|----------------|---------|---------|------|
| | NAD$^+$ | NADP$^+$ | NAD$^+$ | +AdoMet |
| NADPH-CH$_2$-THF | 1130 ± 50 | 1530 ± 40 | |
| CH$_3$-THF-menadione | 2100 ± 70 | 3300 ± 250 | |

* NADPH for HaMTHFR; NADH for all others.

(estimated using an NADP concentration of 10 mM) in Arabidopsis and maize tissue extracts were 14–34 nmol min$^{-1}$ mg$^{-1}$ protein, which would allow significant NADH formation during the oxidoreductase assays. Yeast contained no detectable NADP phosphatase activity (<0.3 nmol min$^{-1}$ mg$^{-1}$ protein).

Metabolism of [methyl-$^{14}$C]CH$_3$-THF—MTHFRs in yeast and mammals are cytosolic enzymes (9), and the lack of NH$_2$-terminal transit sequences (Fig. 1) indicates that plant MTHFRs are likewise cytosolic. If they are, the very low NADH/NAD$^+$ ratios that prevail in plant cytosol (10$^{-3}$) (34) might allow the MTHFR reaction to proceed in the reverse direction. An exploratory test of this possibility was made by supplying a tracer quantity of [methyl-$^{14}$C]CH$_3$-THF to illuminated leaf tissue and analyzing labeled metabolites (Fig. 5, panels A and B). In both Arabidopsis and maize, $^{14}$C was readily metabolized to serine, sugars, and starch. A simple explanation for this labeling pattern is that $^{14}$CH$_3$-THF is oxidized to $^{14}$CH$_2$-THF, allowing $^{14}$C to enter serine via the action of glycine hydroxymethyltransferase (11, 12). From serine, label is expected to flow to photosynthetic end products (17, 35). Consistent with this explanation, when a large dose of serine was given together with $^{14}$CH$_3$-THF, label was trapped in the serine pool (Fig. 5, panels C and D). That the trapping effect was less marked in the C$_2$ plant Arabidopsis may be explained by its high capacity to metabolize serine; measurements showed that ~60% of the serine supplied to Arabidopsis was metabolized during the experiment.

Southern Analyses—Southern analyses were carried out in order to estimate the number of MTHFR genes in Arabidopsis and maize (Fig. 6). For Arabidopsis (Fig. 6, panel A), the sizes and intensities of hybridizing bands indicated two genes, corresponding to the AtMTHFR-1 and -2 cDNAs with respect to the predicted restriction sites. For maize (Fig. 6, panel B), the banding pattern indicated at least two MTHFR genes. Taken with the evidence from the data bases, the Southern analyses show that the cDNAs that we have identified represent both MTHFR genes of Arabidopsis, and what appears to be the most strongly and widely expressed MTHFR gene of maize.

**DISCUSSION**

The identification of cDNAs encoding MTHFR completes the set of plant genes required for the synthesis of methyl groups from serine and formate (12). This opens the way for systematic application of reverse genetics to investigate folate-mediated one-carbon metabolism in plants. It will also permit comprehensive studies of the expression of one-carbon metabolism.
The same can be concluded for the AdoMet response of the plant enzymes, because neither enzymes from plant sources nor recombinant plant MTHFRs were inhibited by AdoMet, whereas the recombinant human enzyme was inhibited. Moreover, the demonstration that recombinant plant MTHFR has intact subunits excludes the possibility that proteolytic cleavage between the catalytic and COOH-terminal domains causes the AdoMet insensitivity. This interdomain cleavage is the most likely origin of artificial AdoMet insensitivity (2).

The lack of inhibition of plant MTHFRs by AdoMet seems most likely to be due to absence of an AdoMet binding site. The presence of an AdoMet binding site is required for optimal activity in mammalian MTHFR. The lack of an AdoMet binding site in plant MTHFR suggests that AdoMet binding is not a prerequisite for optimal activity in plant MTHFR.

Plant MTHFR proteins resemble those of other eukaryotes in having a catalytic domain homologous to the E. coli enzyme, and a long (>270-residue) COOH-terminal extension. Like the mammalian and yeast counterparts, plant MTHFRs appear to be cytosolic proteins inasmuch as they lack obvious targeting sequences. Despite these overall structural similarities, the plant enzymes have the opposite pyridine nucleotide preference to mammalian MTHFR, and are not inhibited by AdoMet. Because of the far-reaching implications of these conclusions for the regulation of plant one-carbon metabolism, it is important to examine the evidence for them. The conclusion that plant MTHFRs are NADH-dependent rests (i) on the properties of three different recombinant enzymes from Arabidopsis and maize (with control experiments in which recombinant human MTHFR expressed in the same system proved to be NADPH-dependent), and (ii) on data for enzymes isolated directly from these and two other plant species. Taken together, this evidence rules out the possibility that the NADH-dependence of the plant enzymes is an artifact of expression in yeast. The same can be concluded for the AdoMet response of the plant enzymes, because neither enzymes from plant sources nor recombinant plant MTHFRs were inhibited by AdoMet, whereas the recombinant human enzyme was inhibited. Moreover, the demonstration that recombinant plant MTHFR has intact subunits excludes the possibility that proteolytic cleavage between the catalytic and COOH-terminal domains causes the AdoMet insensitivity. This interdomain cleavage is the most likely origin of artificial AdoMet insensitivity (2).

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At a pH of 7.6 ([H+] = 2.5 × 10^{-6} M), the cytosolic NAD and NAD concentrations in illuminated spinach leaves have been estimated as 7 × 10^{-7} and 6 × 10^{-4} M, respectively (34). Using these values in Equation 1 gives a value of 1.3 for the CH3-THF/CH2-THF ratio at equilibrium. A value so close to unity connotes a freely reversible reaction in the cytosol (∆G = 0). A physiologically reversible MTHFR reaction could account for the absence of allosteric inhibition by AdoMet in the plant enzymes, since a reversible reaction could maintain an adequate pool of CH2-THF for thymidylate and purine synthesis, without need of a feedback signal from methyl metabolism. Similar considerations may apply to E. coli MTHFR, which is also NADH-dependent and AdoMet-insensitive, as the NADH/NAD ratio is very low in aerobically grown E. coli cells (36). Note that for ready interconversion of CH3-THF and CH2-THF to occur, the thermodynamic reversibility of Equation 1 must be accompanied by kinetic reversibility. Thus, the forward and reverse rates of the MTHFR reaction in vivo would need to be at least as great as those for other reactions forming and consuming CH3-THF and CH2-THF, otherwise the calculated ratio of ~1 would probably not hold. Because the MTHFR activities measured in plant extracts (5–25 nmol min^{-1} mg^{-1} protein) are similar to or higher than those reported for methionine synthase, cytosolic glycine hydroxymethyl transferase and sarcosine dehydrogenase (11, 37), whose sequential activities measured in plant extracts (5–25 nmol min^{-1} mg^{-1} protein) are similar to or higher than those reported for methionine synthase, cytosolic glycine hydroxymethyl transferase and sarcosine dehydrogenase (11, 37), whose sequential action in animal tissues provides a route to convert the methyl group of AdoMet, via formaldehyde, to CH2-THF (9). However, while there are no reports that it occurs in plants, oxidative demethylation of CH3-THF, or of methylated products derived from it, could potentially generate [14C]formaldehyde and hence CH3-THF and [14C]serine. Other caveats are that the (necessarly) large dose of 14CH3-THF used may have perturbed one-carbon metabolism, and that the monoglutamyl form supplied may not have acted as a faithful tracer for endogenous polyglutamylated forms. The direct conversion of 14CH3-THF to 14CH2-THF via MTHFR nonetheless remains the simplest explanation of our 14C-tracer results.

Based on the thermodynamic considerations outlined above, together with the 14CH3-THF metabolism data, we suggest that the MTHFR reaction is reversible in plants. Support for this comes from early work by Clandinin and Cossins (41), who showed that germinating peas converted supplied 14CH3-THF to 5- and 10-[14C]formyl-THF.