The subcellular distribution of Met and S-adenosylmethionine (AdoMet) metabolism in plant cells discloses a complex partition between the cytosol and the organelles. In the present work we show that Arabidopsis contains three functional isoforms of vitamin B12-independent methionine synthase (MS), the enzyme that catalyzes the methylation of homocysteine to Met with 5-methyltetrahydrofolate as methyl group donor. One MS isoform is present in chloroplasts and is most likely required to methylate homocysteine that is synthesized de novo in this compartment. Thus, chloroplasts are autonomous and are the unique site for de novo Met synthesis in plant cells. The additional MS isoforms are present in the cytosol and are most probably involved in the regeneration of Met from homocysteine produced in the course of the activated methyl cycle. Although Met synthesis can occur in chloroplasts, there is no evidence that AdoMet is synthesized anywhere but the cytosol. In accordance with this proposal, we show that AdoMet is transported into chloroplasts by a carrier-mediated facilitated diffusion process. This carrier is able to catalyze the unidirectional uptake of AdoMet into chloroplasts as well as the exchange between cytosolic AdoMet and chloroplastic AdoMet or S-adenosylhomocysteine. The obvious function for the carrier is to sustain methylation reactions and other AdoMet-dependent functions in chloroplasts and probably to remove S-adenosylhomocysteine generated in the stroma by methyltransferase activities. Therefore, the chloroplastic AdoMet carrier serves as a link between cytosolic and chloroplastic one-carbon metabolism.

The sulfur-containing amino acid Met is essential in all organisms as a building block of proteins and as a component of the universal activated methyl donor S-adenosylmethionine (AdoMet). 1 By using the aquatic plant Lemna pausiacostata, Giovanelli et al. 1 have shown that the synthesis of AdoMet accounts for ~80% of Met metabolism, whereas the synthesis of proteins (the only pathway consuming the entire Met molecule) drives ~20% of Met. More than 90% of AdoMet is then used for transmethylation, leading to nucleic acid, protein, lipid, and other metabolite modifications (1). Utilization of the methyl group of AdoMet in transmethylation is accompanied by recycling of the homocysteinyl moity and regeneration of Met, a set of reactions designated as the activated methyl cycle.

Most of the genes and enzymes involved in Met and AdoMet synthesis and metabolism have been characterized in plants (for reviews see Refs. 2–4). One of the most intriguing findings is the complex subcellular distribution of these metabolic routes. The first two reactions specific for de novo Met synthesis consist of the conversion of cysteine into homocysteine (Hcy) by the enzymes cystathionine γ-synthase and cystathionine β-lyase. Both enzymes are present only in the chloroplasts (2). Hcy is then methylated to Met by transfer of the methyl group of 5-methyltetrahydrofolate (5-CH3-H4PteGlu,1), a reaction catalyzed by methionine synthase (MS). There are two types of MS, a cobalamin-dependent enzyme that contains a vitamin B12 cofactor and a cobalamin-independent isoenzyme (5). To date, only the cobalamin-independent MS activity has been described in higher plants (6–9), although cobalamin-dependent enzymes also occur in the photosynthetic protist Euglena gracilis (10). Plant MS seems to be present only in the cytosol (6–9), thus implying that Hcy originating from cysteine has to exit the chloroplast to be converted to Met.

MS not only catalyzes the last reaction in de novo Met synthesis but also serves to regenerate the methyl group of AdoMet after methylation reactions. In plants as well as in eukaryotes, the two other enzymes participating in the activated methyl cycle, namely AdoMet synthetase and S-adenosylhomocysteine (AdoHcy) hydrolase, are most probably present exclusively in the cytosol (2, 3, 11). As a consequence, it is usually considered that chloroplasts and mitochondria must import AdoMet from the cytosol, principally to fulfill methylation reactions. Also, because AdoHcy produced during methylation reactions is a potent competitive inhibitor of methyltransferases, it is assumed that chloroplasts and mitochondria have to import AdoHcy to the cytosol to maintain the AdoMet/AdoHcy ratio that regulates methyltransferase activities (for a review see Ref. 12). In support of these statements, mitochondria isolated from rat liver were found to transport AdoMet from the cytosol via a carrier-mediated system that is inhibited by AdoHcy (13). Recently, mitochondrial carriers capable of...
exchanging cytosolic AdoMet for mitochondrial AdoHcy were cloned and characterized in yeast (14) and human (15). Such a transport system has never been described for plant mitochondria or plastids.

The aim of the present work was to explore the role of chloroplasts in relation to Met and AdoMet metabolism. First, we have shown the existence of a cobalamin-independent MS in the plastids, thus rendering this compartment autonomous for de novo Met synthesis from its precursors cysteine, aspartate, and 5-CH₃-H₄PteGlu. In Arabidopsis, two additional isoforms of B₁₂-independent MS are present in the cytosol and are most probably devoted to the recycling of Met in the course of the activated methyl cycle. Second, we found that AdoMet is transported from the cytosol to chloroplasts by a carrier-mediated facilitated diffusion process. Besides the uniport uptake of AdoMet, the carrier can catalyze the exchange between cytosolic AdoMet and chloroplastic AdoMet or AdoHcy, thus suggesting that it can be involved in the prevention of AdoHcy accumulation in the stroma.

**EXPERIMENTAL PROCEDURES**

**Plant Material—Arabidopsis thaliana** (ecotype Wassilewskija) plants were grown in soil under greenhouse conditions (23°C with a 16-h photoperiod and a light intensity of 200 μmol of photons m⁻² s⁻¹) until harvested for analysis. A. thaliana (ecotype Columbia) cell suspension cultures were grown under continuous white light (40 μmol of photons m⁻² s⁻¹) at 23°C with rotary agitation at 125 rpm in Gamborg’s B5 medium supplemented with 1 μM 2-naphthalene acetic acid and 1.5% (w/v) sucrose. Pea (Pisum sativum L. var. Douce Provence) plants were grown for 9 days under a 12-h photoperiod (140 μmol of photons m⁻² s⁻¹) at 22°C (day) and 20°C (night). Etiolated pea plants were grown in complete darkness at 20°C, and leaves were collected under a green safelight. Freshly harvested spinach (Spinacia oleracea L.) leaves were obtained from a local grower and stored at 4°C until required for use.

**Chemicals**—[6R,6S]-5-Methyl-5,6,7,8-tetrahydropteroyl-1-glutamate acid (5-CH₃-H₄PteGlu) and 1-homocysteine thiolactone were obtained from Sigma. Pteroylpenta-γ-glutamic acid was purchased from Schircks Laboratories (Jona, Switzerland). [carboxyl-¹⁴C]AdoMet (2.18 GBq mmol⁻¹ in 0.2 M H₂SO₄, Amersham Biosciences) was mixed with AdoMet (p-toluene sulfonate salt, Sigma) and neutralized with BaCO₃ as described by Teyssier et al. (16).

**Cloning of AtMS1-3 cDNAs—**Poly(A)⁺ mRNAs were isolated from above-ground parts of 3-week old A. thaliana plants and used to construct a Marathon™ cDNA amplification library (17). Full-length cDNAs were generated by PCR using primers overlapping the region containing the initiation Met codon and the 3’-untranslated region of each gene (Table I). Amplification was done with the Pfu DNA polymerase according to the manufacturer’s instructions (Promega). PCR products were subcloned into the pBluescript KS vector digested with Smal and sequenced (GenomeExpress, Meylan, France). The cloned sequences were designated AtMS1, -2, and -3 for A. thaliana methionine synthase homologs.

**Expression of AtMS Proteins in Escherichia coli**—The coding sequences of the AtMS cDNAs were amplified from pBluescript plasmid templates by high fidelity PCR using Pfu DNA polymerase and primers listed in Table I. For overexpression of AtMS1 and AtMS2, the full-length coding sequences were amplified, digested with BspHI and XhoI sites of pET-23d (Novagen) to give plasmids pET-MS1 and pET-MS2. The pET-MS constructs were introduced first into E. coli DH5α and then into BL21(DE3)pLysS cells (Stratagene). Transformed cells were grown at 37°C in LB medium containing carbenicillin (100 μg ml⁻¹), chloramphenicol (34 μg ml⁻¹), and 0.5 mM ZnSO₄ until A₆₀₀ reached 0.6. Isopropylthio-β-D-galactoside was added (final concentration 0.5 mM), and the cells were further grown for 4 h at 28°C (AtMS1), 16 h at 28°C (AtMS2), or 16 h at 16°C (AtMS3). Subsequent operations were done at 4°C. Cells were pelleted (4,000 × g, 20 min), resuspended in 50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 5% (v/v) glycerol, 1 mM PMSF, and a mixture of protease inhibitors (catalog number 1873580, Roche Applied Science), and disrupted by sonication (Sonifier 250, Branson). The soluble protein extracts were separated from the cell debris by centrifugation at 16,000 × g for 15 min and desalted on PD-10 columns (Amersham Biosciences) equilibrated in 50 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, and 5% (v/v) glycerol.

**Measurement of Cobalamin-independent MS Activity—**Cobalamin-independent MS was assayed using unlabeled 5-CH₃-H₄PteGlu as a substrate, the reaction product tetrahydrofolate being detected spectrophotometrically following its conversion to 5,10-methenyltetrahydrofolo
late by heating with formic acid (18). The standard assays contained in a final volume of 400 µl 10 mM potassium phosphate buffer, pH 7.2, 50 mM Tris-HCl, pH 7.2, 0.1 mM MgSO₄₅, 10 mM dithiothreitol, 2 mM Hcy, 150 µM (6R,6S)-5-CH₂-H₂PteGlu₅ or (6R,6S)-5-CH₂-H₂PteGlu₃, and desalted protein extracts. The reaction was initiated by the addition of Hcy and incubated at 30 °C for 30–40 min. The reaction was stopped by adding 100 µl of 5 N HCl, 60% (v/v) formic acid and heating for 14 min at 84 °C. After equilibration at room temperature, the assays were centrifuged at 16,000 × g for 5 min to remove precipitated proteins. The absorbance of the supernatant was measured at 350 nm, and 5,10-methylenetetrahydrofolate was quantified using an absorption coefficient of 26,500 M⁻¹ cm⁻¹ (18). The Hcy stock solution was prepared by hydrolysis of the Hcy thioiactone, and the actual concentration was determined with 5,5-dithiobis-2-nitrobenzoic acid (18). The standard assays contained, in a final volume of 20 µl using oligo(T)_30 primers (Thermoscript RT-PCR System, Invitrogen). For each real time PCR experiment, 5 µl of DNA preparation was used as a template in a standard 10-µl LightCycler PCR with appropriate primers (Table I) used at a final concentration of 1 µM and 3 mM MgCl₂. Amplification and detection were performed with the following profile: 95 °C for 8 min followed by 40 cycles of 9 s for 10 s, 66 °C for 15 s, and 72 °C for 8 s. Data were analyzed with the LightCycler Relative Quantification software (Roche Applied Science) using the amplification of actin cDNA (GenBank™ accession number U39449, primers listed in Table I) as internal standard of mRNA integrity and cDNA preparation. The specificity of the reaction was verified by melting curve analysis obtained by increasing temperature from 55 to 95 °C (0.1 °C/s).

Measurement of AdoMet Uptake by Intact Chloroplasts—Uptake of AdoMet was measured by silicone oil-filtering centrifugation (27) using spinach leaf chloroplasts purified according to Mouriou and Douce (19). The intactness of the chloroplasts was tested by using an oxygen electrode, and was found to be close to 95%. Chlorophyll content was determined as described by Bruinsma (29). Polypropylene tubes (0.4 ml) were filled with 20 µl of 1 mM HClO₄, 70 µl of AR200 silicone oil (Wacker-Chemie GmbH, Munich, Germany), and 140 µl of medium A (0.33 mM sorbitol, 50 mM Hepes-KOH, pH 7.2) containing various concentrations of the radiolabeled substrate. Uptake was started by addition of 10 µl of freshly prepared chloroplasts (80–100 µg of chl/assay) using a small plastic spatula to achieve rapid dispersion of the chloroplasts in the medium. After incubation for 10–500 s at 25 °C under low light conditions, uptake was stopped by centrifuging the tubes at 3,000 × g for 2 min (swinging bucket, Kubota RM-15200 microcentrifuge). Aliquots of the supernatant were taken for determination of substrate-specific radioactivity, and a 10-µl aliquot of the HClO₄ phase was taken for determination of radioactivity incorporated into chloroplasts. The kinetics of AdoMet uptake were fitted to a single exponential by using Equation 1, where A₀ and A are the amount of AdoMet incorporated in the chloroplasts at time t and at the equilibrium, and kₘₐₜ is the pseudo-first order rate constant.

\[ A_t = A_0 \left(1 - \exp \left(-k_{\text{ma}}t \right) \right) \]

(1) To analyze AdoMet/AdoHcy counter-exchanges across the chloroplast envelope, freshly prepared chloroplasts were loaded with AdoMet or AdoHcy by incubation in the dark with 100 µM unlabeled substrates for 1 h at room temperature. The chloroplasts were quickly cooled down on ice for 10 min and then washed twice by dilution with ice-cold medium A and centrifugation at 2,500 × g for 3 min. The pellet chloroplasts were gently resuspended in medium A and analyzed for their ability to import 100 µM [carboxy-14C]AdoMet, as described
Arabidopsis Contains Three Isoforms of B12-independent MS—The first goal of our study was to identify and characterize all the genes that may encode MS in Arabidopsis, in particular regarding the occurrence of an Hcy-methylating enzyme in the chloroplast. A search in the Arabidopsis Information Resource database (www.arabidopsis.org) revealed three genes coding putative cobalamin-independent MS in the Arabidopsis genome (Arabidopsis Genome Initiative entries At5g17920, At3g03780, and At5g20980). The full-length cDNAs were obtained by RT-PCR and designated AtMS1, AtMS2, and AtMS3, respectively. The AtMS1 and AtMS2 cDNAs encode 765-residue (~84 kDa) polypeptides that are 92% identical to each other and 87–89% identical to other known B12-independent MS from higher plants. The deduced AtMS3 protein (812 residues, ~99 kDa) shares 79–81% identity with AtMS1, AtMS2, and other known plant MS. A summary of the primary sequences alignment using a similarity tree (Fig. 1) clearly indicates that AtMS3 is apart from other plant cobalamin-independent MS proteins identified so far. When compared with B12-dependent MS from other organisms, the AtMS proteins are 49% identical to MetE from E. coli, 47% identical to Met6p from Saccharomyces cerevisiae, and 41% identical to B12-independent MS from Chlamydomonas reinhardtii (Fig. 1).

To prove that the AtMS clones encode functional B12-independent MS enzymes, the three Arabidopsis cDNAs were subcloned into the expression vector pET-32d and introduced into the E. coli strain BL21(DE3)pLysS. Overexpression of AtMS1 and AtMS2 led to soluble gene products comprising up to 30% of the total cellular proteins. AtMS3 was expressed in E. coli as a truncated protein with an N-terminal 48-residue deletion. The expressed protein was not an abundant protein in crude extracts, but its presence in the soluble fraction was assayed by Western blot experiments (data not shown). Enzyme activity was determined using the non-radioactive assay described by Drummond et al. (18) with (6R,8S)-5-CH3-H4PteGlu5 as a substrate. By using our standard incubation conditions, the bacterial endogenous MetE activity was negligible compared with the activity measured when the plant proteins were expressed. As shown in Table II, the three Arabidopsis proteins are functional B12-independent MS capable of methylating Hcy to Met with 5-CH3-H4PteGlu5 as methyl donor. The ~40-fold difference in activities between AtMS1 and AtMS2 on the one hand and AtMS3 on the other hand is because of the relative abundance of the proteins in the E. coli crude extracts. The apparent Kₘ values for 5-CH3-H4PteGlu5 were ~60 μM for the AtMS1 and AtMS2 enzymes, and 17 μM for AtMS3 (Table II). The substrate specificity of the Arabidopsis B12-independent MS enzymes was further analyzed using the monoglutamate form of 5-CH3-H4PteGlu5, as methyl donor. As shown in Table II, 5-CH3-H4PteGlu5 cannot replace efficiently the polyglutamate substrate, a ratio of at least 200:1 in favor of the pentaglutamate being observed for AtMS1 and AtMS2. These properties are consistent with previous reports (8, 31–32) showing that the cobalamin-independent MS from E. coli, yeast, or higher plants are unable to accept 5-CH3-H4PteGlu5 as methyl donor.

MS Isoforms Are Present in the Cytosol and the Chloroplasts—AtMS3 is the only B12-independent MS described in higher plants that is predicted to possess a targeting sequence. Indeed, analysis of the N-terminal extension of AtMS3 using the prediction softwares TargetP (33) and Predotar (version 0.5; www.inra.fr/predotar/) revealed features typical for mitochondrial or chloroplastic transit peptides, thus suggesting that this protein is targeted to the organelles. To verify this prediction, we first analyzed the subcellular distribution of B12-independent MS isoforms in Arabidopsis by Western blot. A preliminary experiment indicated that antibodies rose against AtMS1 cross-reacted with both AtMS2 and AtMS3 recombinant proteins (data not shown). As shown in Fig. 2, total soluble extracts from Arabidopsis or pea leaves contain two polypeptides of 84 ± 1 and 81 ± 1 kDa, respectively, which react with the antibodies. The occurrence of these polypeptides was then analyzed in purified chloroplasts and mitochondria and in a cytosolic enriched fraction (see “Experimental Procedures”). Fig. 2 clearly indicates that the high molecular weight MS polypeptide is detected in the cytosolic enriched fraction but not in the organelar fractions. In Arabidopsis, the low molecular weight MS polypeptide was detectable in soluble proteins obtained from purified chloroplasts (stroma) but was absent in purified mitochondria (matrix). This immunological pattern was confirmed with organelles purified from pea plants developed in light or in the dark (Fig. 2; the faint band detected in mitochondria from etiolated leaves is attributable to a cross-contamination by plastids). Thus, these results indicate that B12-independent MS is present in both the cytosol and the stromal space of plastids (chloroplasts and etioplasts).

To analyze the subcellular localization of the AtMS proteins in vivo, the N-terminal regions (approximately one-third of the proteins) were fused upstream to the GFP marker protein. As shown in Fig. 3, the expression of AtMS1-GFP and AtMS2-GFP in Arabidopsis protoplasts resulted in green fluorescence throughout the cytoplasm and the nucleus, a pattern similar to the one observed with GFP alone. These results demonstrate
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Activity measurements were made at 30 °C using desalted crude extracts obtained from E. coli BL21(DE3)pLysS cells expressing the AtMS1, AtMS2, or AtMS3 cDNAs. For activity determinations, the 5-CH$_3$H$_4$PteGlu$_5$ substrate was used at a final concentration of 150 μM. For the determination of the $K_m$ values for 5-CH$_3$H$_4$PteGlu$_5$, the Hcy concentration was set at 2 mM. Data are means of three replicates ± S.D. ND, not detected.

| Activity with 5-CH$_3$H$_4$PteGlu$_5$ (nmol min$^{-1}$ mg$^{-1}$ protein) | AtMS1 | AtMS2 | AtMS3 |
|-------------------------------------------------------------|-------|-------|-------|
| Activity with 5-CH$_3$H$_4$PteGlu$_5$ (nmol min$^{-1}$ mg$^{-1}$ protein) | 26.5 ± 3.7 | 20.7 ± 1.5 | 0.6 ± 0.1 |
| $K_m$ for 5-CH$_3$H$_4$PteGlu$_5$ (μM) | ≤0.1 | ≤0.1 | ND |

The faint immunolabeling of the low molecular weight MS isoform in the mitochondrial extract from etiolated pea leaves is because of a cross-contamination of this fraction by etioplasts (10–15% as judged by carotenoid measurements).

**Fig. 2.** Western blot analysis of MS isoforms in subcellular fractions from Arabidopsis and pea. Total soluble extracts, purified plastids and mitochondria, and cytosolic-enriched fractions were obtained from Arabidopsis or pea plants as described under "Experimental Procedures." Proteins were separated by SDS-PAGE using 10% acrylamide gels, transferred to nitrocellulose membranes, and probed with the antibodies raised against AtMS1. The amounts of proteins loaded on the gels are 50 μg for the total extracts (T), 30 μg for the chloroplast (Ch) or etioplast (Et) stroma, 20 μg for mitochondrial matrix (M), and 20 μg for the cytosolic enriched fraction (Cy). The faint immunolabeling of the low molecular weight MS isoform in the mitochondrial extract from etiolated pea leaves is because of a cross-contamination of this fraction by etioplasts (10–15% as judged by carotenoid measurements).

**Fig. 3.** Expression of GFP fused to the N-terminal regions of the AtMS proteins in Arabidopsis protoplasts. Constructs encoding fusion proteins between the N terminus of AtMS isoforms and the engineered reporter protein sGFP/S65T) were introduced into Arabidopsis protoplasts as described under "Experimental Procedures." GFP (green pseudo-color) and chlorophyll (red pseudo-color) fluorescence was observed by fluorescence microscopy using a Zeiss Axioplan 2 microscope. Scale bar, 10 μm.

that AtMS1 and AtMS2 are cytosolic proteins. Expression of the AtMS3-GFP fusion protein in protoplasts resulted in a pattern of green fluorescence that colocalized with the red autofluorescence of chlorophyll (Fig. 3). These data indicate that AtMS3 contains a functional plastid targeting sequence, a result in good agreement with the Western blot analysis shown in Fig. 2. It should be noted that a small part of protoplasts transformed with AtMS3-GFP displayed green fluorescence in chloroplasts and in punctate structures scattered throughout the cytosol (data not shown). These small particles were identified as mitochondria. The physiological significance of this last finding is not known because Western blots presented in Fig. 2 clearly indicate that mitochondria do not contain a B$_{12}$-independent MS isoform. Also, the AtMS3-GFP fusion protein is under the control of the cauliflower mosaic virus 35S promoter and thus very likely expressed at a high non-physiological level in protoplasts, a situation that may disturb the sorting machinery.

Cytosolic and Chloroplastic MS Expression Patterns—To have insight into the physiological function of the three MS isoforms, we examined the expression of the atms genes in different organs from Arabidopsis by quantitative real time RT-PCR. As shown in Fig. 4, the atms1, atms2, and atms3 mRNAs were detected in all analyzed organs (roots, stems, leaves, flowers, siliques, and seeds), demonstrating that the corresponding genes were transcriptionally active. In the growth conditions used, the three genes displayed, however, considerably different steady-state levels and expression profiles. Indeed, the atms1 mRNA coding the first cytosolic MS isoform was the most abundant transcript in all organs, with the highest level in flowers and the lowest level in roots. The atms2 mRNA coding the second cytosolic MS isoform largely followed the atms1 mRNA expression pattern and was generally 4–10-fold less abundant than atms1, with the exception of flowers where the ratio is 80:1 in favor of atms1 (Fig. 4A). The atms3 gene displayed a fairly constant and very low expression level in stems, leaves, flowers, and siliques. In these organs the mRNA coding the plastidial MS isoform is 65–430-fold less abundant than the mRNAs coding the cytosolic isoforms. The ratio is less marked in roots and in dry seeds where the steady-state level of atms3 mRNA is 2.4-fold lower than atms1 and 4-fold higher than atms2 (Fig. 4A).

To analyze the correlation between mRNA and protein abundance, Western blots were realized using soluble protein extracts obtained from the different organs. As mentioned above the 81-kDa polypeptide detected by Western blot corresponds to the chloroplastic AtMS3 protein, whereas the 84-kDa band most probably corresponds to the two cytosolic proteins that are not distinguishable by size. To make sure that both AtMS1 and AtMS2 are present in the high molecular weight band, we separated a soluble protein extract from Arabidopsis cells by two-dimensional gel electrophoresis, identified the cytosolic protein(s) by Western blot, and analyzed the protein spot of interest by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy. The obtained peptide mass map indicated that both AtMS1 and AtMS2 proteins are present in the protein spot, thus indicating that the immunolabeling of the 84-kDa band is representative of the two cytosolic MS isoforms. Comparison of the mRNA and protein expression patterns in Fig. 4, A and B, indicated that there is no direct relationship between the accumulation of MS isoforms and the transcription level of the atms genes. Indeed, roots contain the lowest level of mRNAs coding AtMS1 and AtMS2 but display a large amount of the cytosolic enzymes. On the contrary, dry seeds and more particularly flowers contain high...
levels of the atms1 and atms2 transcripts, but the corresponding proteins do not accumulate. A similar variation between mRNA and protein levels is also observed for AtMS3. With the exception of seeds, the mRNA coding the chloroplastic MS is present at very low levels in all organs, but the immunolabeling of the protein is as strong as the one for the cytosolic isoforms (Fig. 4). The opposite situation is observed in dry seeds which is indicative of a carrier-mediated transport (Fig. 5).

**Chloroplasts Import AdoMet by a Facilitated Diffusion Process**—Although the above-mentioned results indicate that Met synthesis can occur in chloroplasts, there is no evidence that AdoMet is synthesized anywhere but the cytosol. This observation along with the presence of AdoMet-dependent reactions in chloroplasts leads to the proposal that chloroplasts have to import AdoMet from the cytosol. To test this hypothesis, we analyzed AdoMet uptake by intact spinach leaf chloroplasts using silicone oil-filtering centrifugation. Fig. 5A shows the uptake kinetics obtained for various concentrations of [carboxyl-14C]AdoMet. This figure indicates that AdoMet was taken up very rapidly by chloroplasts and leveled off after 2–4 min under our experimental conditions. Fig. 5A also indicates that the final concentration of AdoMet reached in the stroma at the equilibrium is proportional to the concentration of AdoMet initially present in the incubation medium. We ascertained that the radioactive product incorporated into chloroplasts was AdoMet by co-migration with authentic [carboxyl-14C]AdoMet using thin layer chromatography (data not shown). This analysis also indicated that under our experimental conditions [carboxyl-14C]AdoMet was not used for methylation reactions in the chloroplast because [carboxyl-14C]AdoHcy could not be detected. A plot of the initial rate of AdoMet uptake versus substrate concentration in the medium shows a saturable curve, which is indicative of a carrier-mediated transport (Fig. 5B). From these data the calculated apparent $K_m$ and $V_{max}$ values were 38 $\mu$M and 6.3 nmol mg$^{-1}$ chl min$^{-1}$ at 25 °C, respectively. To investigate whether the transport of AdoMet across the chloroplast envelope is energy-dependent, uptake experiments were performed either in light or in darkness, or with or without ATP-Mg. These treatments had no effect on the kinetic parameters of AdoMet uptake (results not shown), thus indicating that ATP hydrolysis or light-induced transmembrane proton potential across the thylakoid membranes was not involved. Thus the uptake of AdoMet into chloroplasts is mediated by a facilitated diffusion process.

**AdoMet Uptake into Chloroplasts Is Inhibited by AdoHcy**—The possibility that transport of AdoMet is mediated by the chloroplastic adenylate carrier, an antiport that exchanges cytosolic for stromal adenylic nucleotides, was ruled out because ATP (up to 1 mM) had no effect on the uptake of AdoMet. The addition of either Met or Hcy (up to 100 $\mu$M) to the external medium did not lead to any modification of the kinetic parameters of the AdoMet carrier (results not shown). Our results...
argue therefore against the proposal that AdoMet is transported by putative Met or Hcy carriers. On the other hand, the AdoHcy molecule that is formed during the course of AdoMet-dependent methylation reactions was found to be a potent inhibitor of AdoMet uptake into chloroplasts. The uptake of AdoMet was measured in the presence of various concentrations of AdoHcy, and the Dixon plot presented in Fig. 6 shows that the inhibition is competitive. The apparent $K_i$ value calculated from this Dixon plot was 14 $\mu$M.

Fig. 6. Inhibition of AdoMet uptake into chloroplasts by AdoHcy. Kinetics of [carboxyl-$^{14}$C]AdoMet uptake into chloroplasts were done with various concentrations of radioactive AdoMet and unlabeled AdoHcy. The initial rates of AdoMet uptake were measured and plotted against AdoHcy concentration for the three AdoMet concentrations indicated. The $K_i$ value for AdoHcy calculated from this Dixon plot is 14 $\mu$M.

were attributable to the unidirectional transport of AdoMet or AdoHcy that occurred during the course of chloroplast washing (the efflux is considerably reduced when this procedure is carried out at low temperature). The pre-loading of chloroplasts with either AdoMet or AdoHcy led to a marked stimulation of [carboxyl-$^{14}$C]AdoMet uptake into the chloroplasts (Fig. 7B). Under standard conditions (25 °C) the influx is so rapid that it was difficult to measure accurately the initial rate of [carboxyl-$^{14}$C]AdoMet uptake into pre-loaded chloroplasts. Decreasing the temperature from 25 to 4 °C led to a marked decrease in the maximum rate of AdoMet uptake so that we could determine that chloroplasts pre-loaded with AdoMet and AdoHcy took up external AdoMet $-10$ and $-6$ times more rapidly than untreated chloroplasts, respectively. These results indicate that external AdoMet is taken up by chloroplasts in counter-exchange with AdoMet or AdoHcy.

DISCUSSION

The subcellular compartmentation of the final step of Met synthesis in plants is still a matter of questioning and debate more than 3 decades after the first report of MS activity in plant extracts (34). This enzyme catalyzes a unique reaction, the methylation of Hcy to Met with 5-CH$_3$H$_4$PteGlu$_n$ as methyl group donor, but is required for two functions, namely to ensure de novo synthesis of Met from Hcy produced in chloroplasts and to regenerate the methyl group of AdoMet following transmethylation reactions. It has been speculated that this dual function may be catalyzed by MS isoenzymes with distinct subcellular locations and possibly distinct catalytic mechanisms, i.e. vitamin B$_{12}$-dependent and -independent activities (2). A survey of the Arabidopsis genome indicated that there is no plant homolog of the B$_{12}$-dependent MS found in bacteria, mammals, or the photosynthetic protozoa E. gracilis. Arabidopsis contains, however, three functional isoforms of the B$_{12}$-independent enzyme, one of which is located in plastids (Figs. 3 and 4). This result suggests strongly that this isoform is devoted to de novo synthesis of Met and that these organelles are autonomous for the synthesis of Met (Fig. 8). The two other MS isoforms in Arabidopsis are located in the cytosol and most probably are dedicated to the regeneration of Met, because this compartment contains the two other key enzymes of the activated methyl cycle, namely AdoMet synthetase and AdoHcy hydrolase (Fig. 8).

The plastid localization of AtMS3 is consistent with the fact that the chloroplast is the site of Hcy synthesis through the trans-sulfuration pathway (2) and that this organelle contains a pool of 5-CH$_3$H$_4$PteGlu$_n$, the only fate of this compound is the synthesis of Met. This compartmentation is also in accordance with the subcellular location of the enzyme polyglutamate synthetase that is involved in the synthesis of the glutamate tail of tetrahydrofolate coenzymes (17). The presence of this enzyme in plastids is of crucial importance because the chloroplastic MS, similarly to the cytosolic isoforms, cannot efficiently utilize the monoglutamate form of 5-CH$_3$H$_4$PteGlu$_n$ as a methyl donor for Hcy methylation (Table II). The facts that 5-CH$_3$H$_4$PteGlu$_1$ was routinely used to measure MS activity in plant extracts and that this substrate replaces laboriously the polyglutamate form of 5-CH$_3$H$_4$PteGlu$_n$ are probably the reasons why chloroplastic MS activity has never been detected (see for example Ref. 37). Finally, the presence of MS in plastids is supported by the observation that isolated chloroplasts incubated with either $^{13}$C-aspartate or $^{35}$S$\text{SO}_4^{2-}$ can synthesize radiolabeled Met (38, 39).

Both the de novo synthesis of Met in plastids and its recycling in the cytosol are ubiquitous reactions in the plant cell. Indeed, the different MS isoforms could be detected by Western blot in all the Arabidopsis organs examined, namely roots,
stems, leaves, flowers, siliques, and seeds. Also, the plastidial and cytosolic proteins could be identified in both green and etiolated pea leaves, thus indicating that their accumulation is independent on light. As noticed previously in *Catharanthus roseus* cell cultures (8) and potato plants (9, 40), we found that there is not always a good correlation between the level of the mRNAs coding the three MS isoforms in *Arabidopsis* and the abundance of the corresponding proteins (Fig. 4). Thus, the steady-state mRNA level for one isoform can vary up to 2 orders of magnitude between two organs with only moderate alteration of the protein level. Similarly, Nikiforova *et al.* (40) reported that the significant reduction in MS mRNA levels in potato plants submitted to a drought stress was not accompanied by modifications of the protein level. Taken together, these
data indicate that the accumulation of MS polyepitides in the cytosol and chloroplasts is tightly controlled at both the transcriptional and post-transcriptional levels in order to maintain the activities of de novo Met synthesis and the activated methyl cycle. Another example of the tight control of Met synthesis and recycling concerns the germination process. A proteomic analysis of Arabidopsis seed germination indicated that the cytosolic AtMS1 isoform was present at low levels in dry mature seeds, and its level increased 4-fold after 1 day of imbibition (41). We found that the cytosolic and chloroplastic MS isoforms are present at low level in dry seeds (Fig. 4B) and that the amount of both proteins increased in 1-day imbibed seeds (data not shown). It is conceivable that the activities of transcriptional and post-transcriptional levels in order to maintain the one-carbon metabolism and as a key element in the regulation of the synthesis of the aspartate-derived amino acids in the chloroplast.

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