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Mitochondria Are a Major Site for Folate and Thymidylate Synthesis in Plants*

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The subcellular distributions of folate and folate-synthesizing enzymes were investigated in pea leaves. It was observed that the mitochondrial folate pool (~400 μM) represented ~50% of the total pool. Furthermore, all the enzymes involved in tetrahydrofolate polyglutamate synthesis were present in the mitochondria. In marked contrast, we failed to detect any significant activity of these enzymes in chloroplasts, cytosol, and nuclei. The presence of the tetrahydrofolate synthesis pathway in mitochondria is apparently a general feature in plants since potato tuber mitochondria also contained a high folate concentration (~200 μM) and all the enzymes required for tetrahydrofolate polyglutamate synthesis.

The specific activities of tetrahydrofolate-synthesizing enzymes were rather low (1.5–15 nmol h^{-1} mg^{-1} matrix protein), except for dihydrofolate reductase (180–500 nmol h^{-1} mg^{-1} matrix protein). Dihydrofolate reductase was purified to homogeneity. The enzyme had a native molecular mass of ~140 kDa and was constituted of two identical 62-kDa subunits. Interestingly, this mitochondrial protein appeared to be a bifunctional enzyme, also supporting thymidylate synthesis. The cell distribution of thymidylate synthase was also investigated. No significant activity was observed in cell fractions other than mitochondria, indicating that plant cell mitochondria are also a major site for thymidylate synthesis.

In all organisms, one-carbon transfer reactions are mainly mediated by tetrahydrofolate polyglutamate coenzymes (1). As a result, a number of pathways such as those involved in the metabolism of methionine, serine, purine, or thymidylate are dependent on an endogenous supply of these coenzymes (2). Because animals lack the first three steps of folate biosynthesis, folate supply in these organisms is dependent on feeding. In contrast, plants and microorganisms are able to synthesize tetrahydrofolate *de novo*. This pathway requires the sequential operation of five enzymes: a dihydropterin pyrophosphokinase (HPPK),[1] a dihydropteroate synthase (DHPS), a dihydrofolate synthetase (DHFS), a dihydrofolate reductase (DHFR), and a folylpolyglutamate synthetase (FPGS) (Fig. 1). The second step of this pathway is the target of antimicrobial sulfonamide drugs, and considerable effort has been focused on the molecular characterization of the bacterial enzymes involved in the early steps of folate synthesis (3–5). In plants, the study of folate metabolism is complicated by the presence of different subcellular compartments. Indeed, there is now some evidences that, in addition to the cytosol, discrete pools of folate are associated with mitochondria and chloroplasts (6). The distribution of folate-synthesizing enzymes between the cytosol, mitochondria, and plastids remains, however, uncertain, and it is not clear whether all these compartments are autonomous for folate synthesis. The HPPK and DHPS activities were reported to be carried on a single bifunctional enzyme in plants (7) and in *Plasmodium* (8). This situation is different in bacteria, where two separate proteins are involved (3, 9). Fractionation studies in pea seedlings suggested the presence of the enzyme in a "mitochondrial" fraction (73%) and in a "soluble" fraction (25%) (10), but an eventual cross-contamination of these fractions was not investigated. Likewise, DHFS from pea seedlings was reported to be present in a mitochondrial fraction (40%), a "chloroplastic" fraction (15%), and a soluble fraction (22%), but the cross-contamination of the fractions was not estimated (11). DHFR in plants was described either as a bifunctional enzyme, also supporting the thymidylate synthase (TS) activity (12, 13), or as a monofunctional polypeptide associated with TS in a large multimeric enzyme complex (14). In protozoa, the DHFR and TS activities were also reported to be carried on a single protein, but not in prokaryotes and mammals, where two different proteins are involved (for a review, see Ref. 15). The enzyme has been localized in the cytosol of animal cells (16), but its compartmentalization in plants remains to be established. FPGS was reported to be cytosolic and mitochondrial in mammalian cells (17) and in fungi (18), but its distribution in higher plant cells is still unknown. In bacteria, this enzyme is bifunctional, supporting also the dihydrofolate synthetase activity (5).

Subcellular localization of folate synthesis in higher plant cells is hampered by the difficulty of obtaining large amounts of purified organelles devoid of contamination from the other cell fractions. So far, the only reported studies were focused on the dihydropteroate synthase and the dihydrofolate synthetase (10, 11), but the different cellular fractions were not purified, and the cross-contamination of these fractions was not measured. Furthermore, the distribution of folate (and the distribution of the enzymes involved in its synthesis) might vary from one tissue to another, depending on the specificity of the tissue. From this point of view, the presence in leaf mitochondria of the glycine cleavage system, which represents 40% of the soluble proteins and requires folate as cofactor (19), might affect the cellular folate distribution. In this study, we investigated the presence of the tetrahydrofolate-synthesizing enzymes in

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[1] The abbreviations used are: HPPK, dihydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthetase; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; TS, thymidylate synthase; SHMT, serine hydroxymethyltransferase; HPLC, high pressure liquid chromatography; $H_4PteGlu_n$, 5,6,7,8-tetrahydropteroylglutamate; H_4FGlu_n, 5,6,7,8-tetrahydrofolate with *n* glutamate residues; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate.

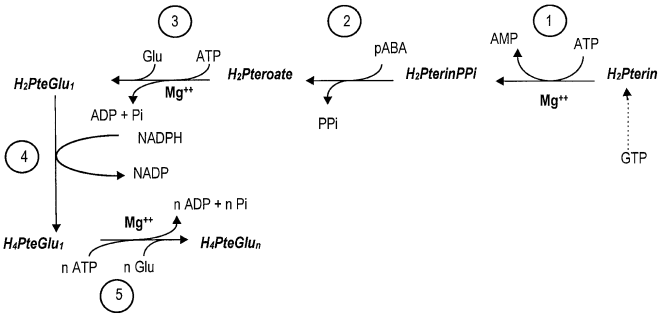

FIG. 1. **$H_4PteGlu_n$ synthesis pathway.** *1*, dihydropterin pyrophosphokinase; *2*, dihydropteroate synthase; *3*, dihydrofolate synthetase; *4*, dihydrofolate reductase; *5*, folylpolyglutamate synthetase. *pABA*, *p*-aminobenzoic acid.

the different cell compartments of pea leaves using Percoll-purified mitochondria, chloroplasts, and nuclei or a cytosol-enriched fraction. In addition, we purified the mitochondrial DHFR/TS bifunctional enzyme, and we studied some of its biochemical properties. We also determined the TS distribution in the leaf cell. Our results indicate that mitochondria are a major site for tetrahydrofolate polyglutamate and thymidylate synthesis in plants.

EXPERIMENTAL PROCEDURES

Materials—Potatoes were purchased at the local market. Pea (*Pisum sativum* L. var. Douce Provence) plants were grown from seeds in vermiculite for 15 days under a 12-h photoperiod at 26 °C (day) or 20 °C (night). Approximately 1 kg of leaves was collected for preparation of the different subcellular fractions. Marker enzymes (fumarase for mitochondria (20), phosphoribulokinase for chloroplasts (21), and phosphoenolpyruvate carboxylase for cytosol (22)) were measured in all the fractions in order to determine any cross-contamination.

Mitochondria were isolated and purified as described previously (23) using a self-generating gradient of Percoll. Chloroplasts were isolated and purified on a discontinuous Percoll gradient as described by Douce and Joyard (24). Protein extracts from these cell organelles were obtained as described previously (25). With these experimental procedures, mitochondria and chloroplasts were usually devoid of contamination from the other compartments.

Nuclei were extracted from pea leaves and purified as described by Dunham and Bryant (26). Purified nuclei were devoid of contamination from mitochondria. Contamination by cytosol proteins was very low (up to 1% of the total protein), but contamination by proteins from chloroplasts was up to 30% of the total protein.

To obtain the cytosol-enriched fraction, leaves (0.5 kg) were ground with a Waring blender at low speed for 3 s in 1 liter of a medium containing 0.3 M mannitol, 20 mM sodium pyrophosphate, pH 7.5, 0.5% (w/v) polyvinylpyrrolidone, 10 mM β-mercaptoethanol, 15 mM malate, 10% (v/v) glycerol, and 1 mM phenylmethylsulfonyl fluoride. After filtration on three layers of gauze, the filtrate was centrifuged at 1500 × *g* to remove cellular debris. The supernatant was centrifuged at 18,000 × *g* to remove all the cell organelles, and the remaining soluble fraction, enriched with cytosol, was concentrated to ~3–5 ml using a Diaflo XM-10 membrane and an Amicon stirred cell.

To obtain the leaf extracts, leaves (0.3 kg) were ground with a Waring blender at high speed for 10 s in 0.5 liter of a medium containing 100 mM potassium P_i, pH 7.5, 0.5% (w/v) polyvinylpyrrolidone, 10 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. After filtration on three layers of gauze, the filtrate was centrifuged at 1500 × *g* to remove cellular debris and then at 18,000 × *g* to remove the membranes. The supernatant was concentrated to a final volume of 5 ml using a Diaflo XM-10 membrane and an Amicon stirred cell.

Folate, pteroate, and pterin were obtained from Sigma. Pterin pyrophosphate and folate polyglutamate were obtained from Schircks Laboratory (Jona, Switzerland). These products were reduced as dihydro and tetrahydro compounds as described by Scrimgeour (27).

Determination of the Folate Content—Folate was determined using a radioisotopic binding assay as described by Waxman and Schreiber (28). The assay medium (100 mM potassium P_i, pH 7, 10 mM β-mercaptoethanol, and 10% (v/v) glycerol) contained 1 μl of [3′,5′,7,9-^{3}H]folic acid (1 μM, 100 GBq mmol^{-1}), various amounts of biological samples, and finally 30 μl of a folate binder (β-lactoglobulin, 10 mg ml^{-1}) in a final volume of 500 μl. After 20 min, the free unbound folate was separated from that which was bound by 10 min of contact with 500 μl of bovine serum albumin-coated charcoal suspension (1.3 g of charcoal were suspended in 100 ml of the assay medium containing 65 mg of bovine serum albumin). Following centrifugation, 600 μl of clear supernatant were added to 15 ml of the scintillation mixture and counted. For each determination, a control was run in the absence of binder to measure the amount of radioactivity that was not adsorbable by charcoal. This remaining radioactivity was subtracted from each measurement. A standard curve was constructed with various amounts (0–20 μl) of 0.4 μM folic acid solution.

Determination of Folate Enzyme Activities—In these measurements, the media and all the solutions were maintained under a stream of argon to minimize the oxidation of folate derivatives. The reactions were assayed at 30 °C. The protein concentrations used in these assays ranged from 5 to 50 mg ml^{-1}, depending of the activity of the samples.

The HPPK activity was estimated in association with the DHPS activity. The standard reaction medium (medium A) contained, in a total volume of 120 μl, 20 mM Tris, 20 mM K_2HPO_4, pH 8, 20 mM β-mercaptoethanol, 15 mM $MgCl_2$, 10 mM ATP, and various amounts of our protein extracts. 2 μl of 2 mM *p*-[*carboxyl*-^{14}C]aminobenzoic acid (1.85 GBq mmol^{-1}) were added to the assay medium, and then the reaction was started by the addition of 100 μM dihydropterin. After 20 min of incubation, the reaction was stopped by heating the samples at 100 °C for 5 min. The samples were centrifuged to remove the precipitated proteins, and the [^{14}C]dihydropteroate formed was determined with a reverse-phase HPLC system (Nova-Pak C_{18} column, Waters) coupled with a Berthold LB 506D scintillation counter. The HPLC conditions were as follows: solvent A, 0.1 M sodium acetate, pH 6; and solvent B, acetonitrile. Solvent B was increased linearly 1% every minute.

The DHPS activity was measured in medium A (final volume of 120 μl) devoid of ATP. 2 μl of 2 mM *p*-[*carboxyl*-^{14}C]aminobenzoic acid (1.85 GBq mmol^{-1}) were added in the assay medium, and then the reaction was started by the addition of 100 μM dihydropterin pyrophosphate. After 5 min of incubation, the reaction was stopped, and the [^{14}C]dihydropteroate formed was estimated as described above.

The DHFS activity was measured in medium A (final volume of 120 μl) in the presence of 3 mM [^{3}H]glutamate (0.5 GBq mmol^{-1}). This medium contained 20 mM K_2HPO_4 buffer. A further addition of K^+ monovalent cation, which is required for DHFS activity (29), did not significantly improve the reaction rate. The reaction was started by the addition of 100 μM dihydropteroate. After 20 min of incubation, the reaction was stopped by the addition of 0.5 ml of a solution containing 0.1 M sodium acetate, pH 5, and 50 mM glutamate. The sample was then loaded on an ion-exchange column (DE52, Whatman) previously equilibrated with the sodium acetate/glutamate solution. The column was washed with 12 ml of this solution, and then the [^{3}H]dihydrofolate formed, which was retained on the column, was eluted with 2 ml of 1 N HCl and counted.

The FPGS activity was determined by the same method as described above, except that the reaction was initiated with 100 μM tetrahydrofolate instead of dihydropteroate.

The DHFR activity was monitored spectrophotometrically by measuring the oxidation of NADPH at 340 nm (ϵ_{mM} = 12.3 cm^{-1}). The assay mixture contained 50 mM potassium P_i, pH 7.2, 20 mM β-mercaptoethanol, 1 mM dithiothreitol, 0.2 mM NADPH, 0.2 mM H_2FGlu_1, and known amounts of protein extract in a final volume of 0.5 ml.

The TS activity was measured by the tritium release assay (30) in 50 mM Tris, pH 7.5, containing 30 mM β-mercaptoethanol, 0.25 mM $H_4PteGlu_n$, 2.5 mM formaldehyde, 100 μM [5-^{3}H]dUMP (0.16 GBq mmol^{-1}), and protein extract in a final volume of 1 ml. At various times, 0.2 ml of the reaction medium was withdrawn and added to 1 ml of a 2% trichloroacetic acid solution containing 0.2 g of charcoal (initially washed with the 2% trichloroacetic acid solution) to remove free dUMP. After vigorous shaking, the suspension was centrifuged, and 0.4 ml of the clear upper phase was counted to quantify ^3H_2O released from [5-^{3}H]dUMP. Activity is expressed as nmol of dUMP formed h^{-1}.

The SHMT activity was measured according to Taylor and Weissbach (31) as described previously (32).

To determine the methylenetetrahydrofolate dehydrogenase activity, known amounts of our protein extract were added to a medium containing 50 mM potassium P_i, pH 7.5, 5 mM NADP, and 2 mM HCHO in a final volume of 500 μl. The reaction was started by the addition of 100 μM tetrahydrofolate, and NADPH formation was monitored spectrophotometrically at 340 nm.

Separation of Mitochondrial Enzymes Involved in Folate Synthesis—Matrix proteins obtained from purified mitochondria (25) were loaded

on a Superdex 200 column (60 × 1 cm, Pharmacia Biotech Inc.) previously equilibrated with the following buffer: 10 mM potassium P_i, 10 mM Tris, pH 7.2, 1 mM EDTA, 1 mM dithiothreitol, 10 mM β-mercaptoethanol, and 15% (w/v) glycerol. The proteins were eluted with the same buffer at 4 °C (rate of elution, 0.3 ml min^{-1}; fraction size, 2.4 ml). To estimate the molecular mass of our enzymes, the P, H, T, and L proteins of the glycine cleavage system present in the matrix extract (19) were used as internal standards to calibrate the column.

Electrophoretic Methods—Under denaturing conditions, electrophoresis was performed on a 1-mm-thick SDS-polyacrylamide slab gel containing a 7.5–15% linear acrylamide gradient. The procedures used were those described by Laemmli (33). Under nondenaturing conditions, electrophoresis was performed without SDS on a 3.5–27% linear acrylamide gradient as described by Clarke and Critchley (34).

Purification of DHFR—Soluble mitochondrial proteins (~1 g) were filtered through a 0.22-μm filter (Sterivex-GS, Millipore Corp.), and ammonium sulfate was added to bring the solution to 30% saturation. After standing for 30 min at 0 °C, the mixture was centrifuged at 15,000 × *g* for 30 min. The pellet was discarded, and more ammonium sulfate was added to the supernatant to bring the solution to 60% saturation. After standing for 1 h at 0 °C, the solution was centrifuged for 30 min at 15,000 × *g*. The pellet was suspended in 8–10 ml of a medium containing 20 mM Tris, pH 7.5, 10 mM 2-β mercaptoethanol, 1 mM dithiothreitol, 1 mM EDTA, and 15% (w/v) glycerol (medium B). The protein suspension was dialyzed against medium B and concentrated by ultrafiltration on a 50-kDa cutoff membrane.

The proteins were loaded on an anionic DEAE-Sepharose column previously equilibrated with medium B at pH 7.5 and connected to a Pharmacia fast protein liquid chromatography system. The column was washed with medium B, at a flow rate of 0.3 ml min^{-1}, until the A_{280} of the effluent was <0.1. Proteins were eluted with a linear gradient of 0–1 M KCl in medium B. Active fractions containing DHFR activity were pooled, dialyzed against medium B, and concentrated by ultrafiltration to 2–3 ml.

In a final step, DHFR was purified by affinity chromatography on a methotrexate-agarose column. The sample was applied to the column previously equilibrated with medium B and connected to the fast protein liquid chromatography system. The flow rate was 0.2 ml min^{-1}. The column was washed until the A_{280} of the effluent was <0.1. The column was eluted with a linear gradient of 0–1 M KCl in medium B. At this stage, the DHFR remained fixed on the column. After washing the column with 12 ml of medium B containing 1 M KCl, the DHFR was finally eluted with 10 ml of a buffer containing 50 mM Tris, pH 8.5, 1 M KCl, 1 mM H_2FGlu_1, 30 mM β-mercaptoethanol, 1 mM dithiothreitol, 1 mM EDTA, and 15% (w/v) glycerol. To avoid oxidation of H_2FGlu_1, this buffer was previously bubbled with argon.

RESULTS

Folate Distribution in Leaf Tissue—The technique used for these measurements is based on a radioassay methodology similar to the one applied for serum and erythrocyte folate determination (28). The results are calculated by direct reference to a standard curve constructed with various concentrations of folate monoglutamate (see "Experimental Procedures"). As shown in Table I, the folate content of pea leaves was ~26 pmol mg^{-1} protein. This corresponds to ~0.5 μg of folate g^{-1} fresh weight, a value in the range of what is generally reported for green vegetables (0.3–3 μg g^{-1}) (6). The folate levels of Percoll-purified chloroplasts and mitochondria are also shown in Table I. In mitochondria, the folate content was ~400 pmol mg^{-1} protein. This value is in agreement with previous measurements (390–1000 pmol mg^{-1} protein) (32) performed with a different method involving zinc HCl treatments and HPLC separation of *p*-aminobenzoyl polyglutamate derivatives (35). Surprisingly, we repeatedly observed that, on a protein basis, the folate level in chloroplasts was ~150 times lower than in mitochondria (Table I). Assuming a mitochondrial volume of 1 μl mg^{-1} protein (36) and a chloroplastic volume of 1.5 μl mg^{-1} protein (37), the mitochondrial and chloroplastic folate concentrations have been estimated to ~400 and 1.7 μM, respectively (Table I). In a leaf extract, the amounts of protein originating from either mitochondria or chloroplasts could be determined through the activity of marker enzymes, fumarase for mitochondria and phosphoribulokinase for chloroplasts (Table I). According to these determinations, it could be calculated that ~50% of the total folate pool was associated with the mitochondria, whereas only 6–7% was associated with chloroplasts. Unfortunately, it was not possible to have a direct reliable estimation of the cytosolic folate content. Indeed, to obtain a cytosol-enriched fraction largely devoid of contamination from the other cell compartments, the grinding of the leaves must be very mild. Under such conditions, the yield of cell aperture was very low, and the cytosol was highly diluted in the grinding medium. During the concentration procedure, folate, in contrast with proteins, was partly lost, preventing any direct determination. However, it might be reasonably assumed that the remaining 40–45% of the folate pool was associated with the cytosol and/or the nuclei. Thus, our results indicate that mitochondria contain the major part of the cellular folate. However, this high folate concentration may reflect a particular situation resulting from the leaf tissue specificity. Indeed, the presence in these mitochondria of the glycine decarboxylase complex and SHMT, which require folate as cofactor and represent 40% of the matrix proteins, could lead to a mitochondrial folate accumulation. To test this hypothesis, we determined the folate content of potato tuber mitochondria, which do not contain glycine decarboxylase activity. As shown in Table I, the folate concentration in these mitochondria was ~200 μM. This concentration, although two times lower than in leaf mitochondria, was still 100 times higher than in chloroplasts. Therefore, taking into account the high folate concentration in mitochondria, we investigated whether all the enzymes required for folate synthesis were present in mitochondria.

TABLE I
Folate pools in pea leaves, pea leaf mitochondria, pea leaf chloroplasts, and potato tuber mitochondria

The folate concentration was calculated assuming a volume of 1 μl mg^{-1} protein for the mitochondrial matrix (36) and 1.5 μl mg^{-1} protein for the chloroplast stroma (37). In the leaf extract, the amounts of protein originating from mitochondria and chloroplasts were estimated through marker enzyme activities: fumarase for mitochondria (specific activity, 850 nmol min^{-1} mg^{-1} protein) and phosphoribulokinase for chloroplasts (specific activity, 450 nmol min^{-1} mg^{-1} protein). The values are the average of four independent determinations.

	Folate content	Folate conc	Protein	Folate distribution
	pmol mg^{-1} protein	*μM*	*%*	*%*
Leaves	26 ± 1.4		100	100
Chloroplasts	2.5 ± 0.5	1.7	65 ± 10	6.3 ± 1
Mitochondria				
Pea	400 ± 50	400	3.5 ± 1	54 ± 15
Potato	200 ± 30	200		

Distribution of Folate-synthesizing Enzymes in Leaf Tissue—The enzyme activities involved in folate synthesis, *i.e.* HPPK, DHPS, DHFS, DHFR, and FPGS, were measured in purified mitochondria, in purified chloroplasts, in purified nuclei, and in a cytosol-enriched fraction. Mitochondrial, chloroplastic, and cytosolic marker enzyme activities were systematically determined in the various fractions to estimate possible cross-contamination. As shown in Table II, all the enzyme activities involved in tetrahydrofolate synthesis were present in pea leaf mitochondria as well as in potato tuber mitochondria. Surprisingly, the DHFR-specific activity was 10–100 times higher than the activities found for the other enzymes, suggesting a possible involvement of DHFR in other metabolic functions. In marked contrast, we failed to detect any significant activity in pea leaf chloroplasts and nuclei. Likewise, in the cytosol-enriched fraction these activities were very low, at the limit of detection, and could be totally accounted for by the small mitochondrial contamination (Table II). In control experiments,

TABLE II

Enzyme activities involved in tetrahydrofolate synthesis in the different pea leaf cell compartments and in potato tuber mitochondria

Fumarase, phosphoribulokinase, and phosphoenolpyruvate carboxylase were marker enzymes for mitochondria, chloroplasts, and cytosol, respectively. In the cytosol-enriched fraction, <1% of the proteins were from mitochondria, and 30–45% were from plastids. Although nuclei were purified on a Percoll gradient, ~25% of the proteins were from chloroplasts, and 1% were from the cytosol. The results presented here are expressed as nmol h^{-1} mg^{-1} protein and are the average of five determinations.

Enzyme	Pea leaves				Potato tuber mitochondria
	Mitochondria	Chloroplasts	Cytosol	Nuclei	
Fumarase	51,000 ± 3000	ND[a]	420 ± 120	ND	
PRK	ND	27,000 ± 2400	10,200 ± 2400	7200 ± 1200	
PEPc	ND	ND[a]	6000 ± 800	60 ± 10	
HPPK + DHPS	1.8 ± 0.3	ND[a]	ND	ND	2 ± 0.4
DHPS	16 ± 3	ND[a]	0.08 ± 0.04	ND	9 ± 2
DHFS	1.6 ± 0.3	ND[a]	ND	ND	2.1 ± 0.4
DHFR	180 ± 50	ND[a]	1.5 ± 0.5	ND	500 ± 100
FPGS	3.5 ± 0.5	ND[a]	0.04 ± 0.03	ND	3.8 ± 0.6
SHMT	2100 ± 240	70 ± 8	190 ± 30	200 ± 30	350 ± 60
MTHFDH	150 ± 20	200 ± 50	180 ± 40	230 ± 50	85 ± 15

[a] ND, not detected; PRK, phosphoribulokinase; PEPc, phosphoenolpyruvate carboxylase; MTHFDH, methylenetetrahydrofolate dehydrogenase.

we verified that these negative results were not the fact of specific inhibitors or proteolytic enzymes. Indeed, the addition of mitochondrial proteins to the same amounts of chloroplastic or cytosolic extract that were used when assayed alone resulted in a good recovery of the added mitochondrial activities (data not shown). Although folate-synthesizing enzymes were not detected in the cytosol, nuclei, and chloroplasts, other folate enzymes were present in these compartments. As shown in Table II, two other folate enzymes, SHMT and methylenetetrahydrofolate dehydrogenase, involved in the interconversion of substituted tetrahydrofolate derivatives, could be easily detected in all the cellular fractions (see also Ref. 38 for SHMT). As previously reported (38), the SHMT activity was very high in leaf mitochondria because of its participation in the photorespiratory pathway. Taken as a whole, these results indicate that folate enzymes are highly compartmentalized and that activities required for tetrahydrofolate polyglutamate synthesis are mainly localized in the mitochondria.

The different activities involved in tetrahydrofolate polyglutamate synthesis in mitochondria were then separated by gel filtration (see "Experimental Procedures"). As shown in Fig. 2, the HPPK and DHPS activities coeluted with an apparent molecular mass of ~300 kDa. This value is higher that the one previously reported for the HPPK/DHPS bifunctional enzyme isolated from pea seedlings (180 kDa) (7). The DHFS activity was eluted with an apparent molecular mass of 54 kDa, in good agreement with a previous report (29). The FPGS activity was eluted with an apparent molecular mass of 70 kDa, a value comparable to those previously reported for plant and mammal FPGS (39, 40). The DHFR activity was eluted with a molecular mass corresponding to ~140 kDa. Similar molecular masses were previously reported for DHFR originating from soybean seedling (41) and carrot cell suspension cultures (42). In protozoa, the apparent molecular mass of the enzyme was also estimated at 150 kDa by gel filtration (43). In this last case, however, DHFR appeared to be a bifunctional protein associated with TS (43). Interestingly, the TS activity in plant mitochondria also coeluted with the DHFR activity (Fig. 2). These results suggest that the DHFR and TS activities from pea leaf mitochondria are also supported by the same enzyme. To verify this, experiments were undertaken to purify mitochondrial DHFR.

Purification of Mitochondrial DHFR/TS—The different steps of purification are summarized in Table III. The DHFR and TS activities coeluted after ion-exchange and affinity chromatographies, thus strengthening the assumption that both activities are supported by the same protein. At the end of the last stage of purification, DHFR/TS was almost pure, giving a single band on SDS gel electrophoresis (Fig. 3). The apparent molecular mass was 62 kDa, which suggests that DHFR/TS is a dimeric protein with two identical subunits (the native molecular mass was estimated to ~140 kDa by gel filtration (Fig. 2)). The final yield of recovery, calculated from Table III, was ~20–25%.

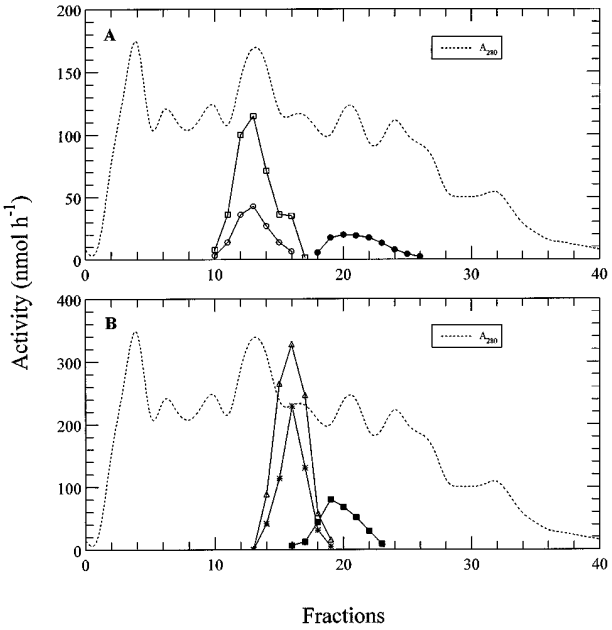

FIG. 2. **Separation by gel filtration of the different enzyme activities involved in tetrahydrofolate polyglutamate synthesis.** *A*, separation of the HPPK (○), DHPS (□), and DHFS (●) activities. To facilitate the graph reading, the DHPS activity was reduced three times. *B*, separation of the DHFR (△), TS (*), and FPGS (■) activities. To facilitate the graph reading, the DHFR activity was reduced 20 times.

Properties of the DHFR/TS Enzyme—As previously observed with the bacterial monofunctional DHFR (44), the reaction catalyzed by the bifunctional plant enzyme presented a lag phase that was considerably shortened by 3–5 min of preincubation of the enzyme with 200 μM NADPH (Fig. 4). According to a previous report (44), this hysteretic behavior might correspond to the time required for the interconversion of an inactive to an active enzyme form. As also shown in Fig. 4, the reaction was completely blocked by trace amounts of methotrexate, a potent inhibitor of DHFR (15). The maximal DHFR activity was obtained for temperatures close to 50 °C, and the optimum pH was between 6.5 and 7.0 (data not shown). Sur-

TABLE III
Purification steps for DHFR and TS from pea leaf mitochondria
The TS activity was measured in the presence of saturated amounts of CH_2-H_4FGlu_5.

Fractions	Total protein	DHFR		TS	
		Activity	Specific activity	Activity	Specific activity
	mg	*nmol h^{-1}*	*nmol h^{-1} mg^{-1}*	*nmol h^{-1}*	*nmol h^{-1} mg^{-1}*
Matrix proteins	434	86,760	200	3422	7.9
$(NH_4)_2SO_4$ (30–60%)	291	79,800	274	3048	10.5
DEAE-Sepharose	25	36,120	1445	1290	51.6
MTX[a]-agarose	0.058	24,180	416,900	864	14,900

[a] MTX, methotrexate.

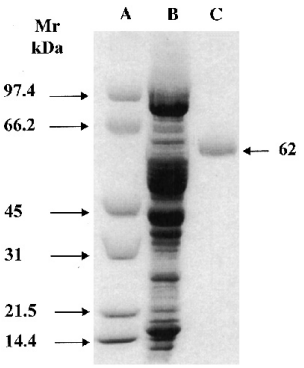

FIG. 3. **SDS-polyacrylamide gel electrophoresis analysis of DHFR/TS purified from pea leaf mitochondria.** *Lane A*, standards; *lane B*, 90 μg of matrix extract; *lane C*, 1 μg of purified DHFR/TS.

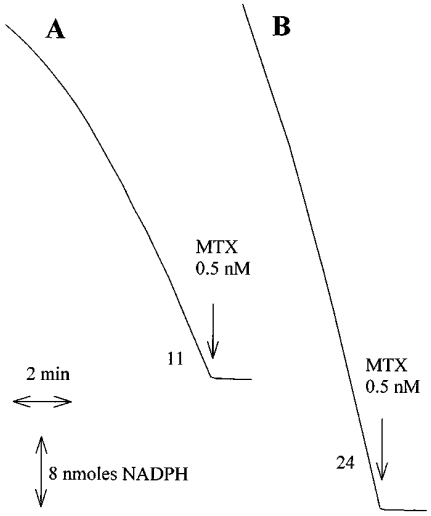

FIG. 4. **H_2FGlu_1 reduction catalyzed by a mitochondrial enzyme extract.** *A*, proteins from the DEAE-Sepharose column (0.7 mg) were added to 500 μl of the reaction medium (see "Experimental Procedures"), and the reaction was immediately initiated by adding H_2FGlu_1. The DHFR activity was monitored at 30 °C by measuring the rate of NADPH consumption. *B*, the proteins were incubated for 5 min in the presence of NADPH before the reaction was initiated by adding H_2FGlu_1. *Numbers* along the lines are nmol of NADPH consumed min^{-1} mg^{-1} protein. *MTX*, methotrexate.

prisingly, at pH 7.5, a value representative of the mitochondrial matrix pH, the activity represented only half of the maximal velocity. The Michaelis constants for NADPH and dihydrofolate monoglutamate were 28 ± 5 and 0.7 ± 0.2 μM, respectively. Interestingly, the K_m values for dihydrofolate monoglutamate and pentaglutamate were very similar (Table IV).

In contrast to DHFR activity, TS activity exhibited no lag phase, but slowly declined with time, even in the presence of saturating amounts of dUMP (100 μM) and CH_2-H_4FGlu_5 (100 μM) (Fig. 5). However, in the presence of 1 mM NADPH, the reaction appeared linear for at least 90 min. These results strongly suggest that the time-dependent inhibition was the result of H_2FGlu_5 accumulation. Indeed, in the presence of NADPH, H_2FGlu_5 produced by TS activity could be recycled into H_4FGlu_5 owing to the associated DHFR activity. The addition of 1 μM FdUMP, a potent inhibitor of monofunctional thymidylate synthase (45), completely blocked the reaction (Fig. 5). The maximal TS activity was obtained for temperatures close to 40 °C, and the optimum pH was between 7 and 7.5 (data not shown). The affinity constants for dUMP and CH_2-H_4FGlu_1 (assuming that only half of the CH_2-H_4FGlu_1, the pro-*R* form, interacted with the enzyme) were 1.5 ± 0.3 and 22 ± 2.5 μM, respectively. In contrast to DHFR, increasing the polyglutamate chain length resulted in a considerable decrease in the K_m of CH_2-H_4FGlu_n for TS (Table IV). The V_{max} of the reaction was also affected by the length of the polyglutamate chain and increased ~3 times when the number of glutamates increased from one to five (Table IV).

The activity ratio of DHFR to TS was generally between 20 and 30. However, we observed that, during storage, this ratio could increase considerably because of instability of the catalytic domain responsible for TS activity. In this connection, it is interesting to note that the domain of the enzyme responsible for TS activity was much more sensitive to protease action than the DHFR domain. Indeed, as shown in Fig. 6, when partially purified enzyme was incubated at 5 °C in the presence of trypsin (20 $\mu g\ ml^{-1}$), TS activity rapidly declined and dropped to 0 after 30 min. In contrast, during the same period, DHFR activity was unaffected and remained constant even after 2 h of incubation. This last observation strongly supports the idea that the two domains catalyzing the DHFR and TS activities are spatially distinct. This assumption is also reinforced by the observation that FdUMP, a potent inhibitor of TS activity, did not affect DHFR activity (data not shown).

Distribution of TS—In animal cells, TS is a monofunctional enzyme localized in the cytosol and/or nuclei (46). To determine whether such an enzyme was also present in plant cells, TS activity was tested in the various cell compartments. As shown in Table V, no activity could be detected in the different cell fractions, except for the mitochondria, where, as described above, it was associated with DHFR activity.

DISCUSSION

The results presented here indicate, for the first time, that higher plant mitochondria are a major site for tetrahydrofolate synthesis. Indeed, we observed that the largest part of the cellular folate and all the enzymes required for tetrahydrofolate synthesis were located in this compartment. Surprisingly, we were not able to detect these enzymes in chloroplasts, nuclei, or cytosol. Although it is difficult to ascertain negative results, it is clear that these activities, if present, were much lower in these last three compartments than in mitochondria. These observations are in agreement with previous reports indicating that a large part of the DHPS and DHFS activities is localized in pea seedling mitochondria (10, 11). Some minor

TABLE IV

Affinity constants of H_2FGlu_n and CH_2-H_4FGlu_n and maximal velocities for purified DHFR and TS

Shown are the effects of polyglutamate chain length on these kinetic parameters. The K_m for CH_2-H_4FGlu_n was calculated assuming that only half of the initial CH_2-H_4FGlu_n, the pro-*R* form, participated in the reaction.

Glu_n	DHFR		TS	
	K_m H_2FGlu_n	V_{max}	K_m CH_2-H_4FGlu_n	V_{max}
	μM	$\mu mol\ min^{-1}\ mg^{-1}$	μM	$\mu mol\ min^{-1}\ mg^{-1}$
Glu_1	0.7 ± 0.2	6.5 ± 1	22 ± 5	0.1 ± 0.03
Glu_5	1.1 ± 0.3	6.5 ± 1	3.5 ± 0.5	0.3 ± 0.1

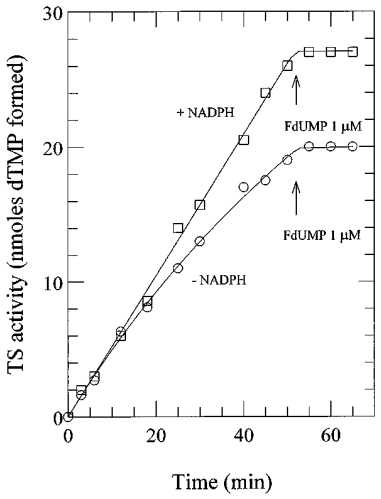

FIG. 5. **Thymidylate synthesis catalyzed by a mitochondrial enzyme extract.** Proteins from the DEAE-Sepharose column (0.65 mg) were added to 2.5 ml of the reaction medium containing 100 μM (6*R*,6*S*)-CH_2-H_4FGlu_5. At each time point, 200 μl were withdrawn to determine the amount of dTMP formed (see "Experimental Procedures"). In the presence of NADPH, the reaction was maintained linear for at least 50 min.

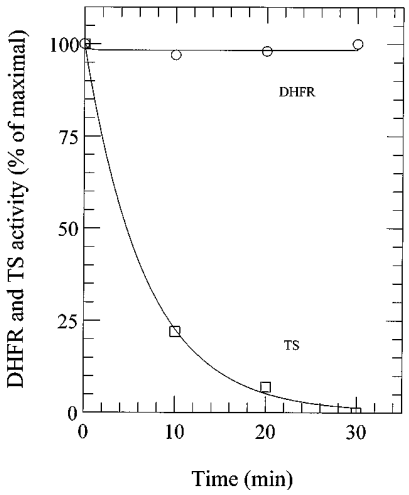

FIG. 6. **Effect of trypsin on the DHFR and TS activities.** The DHFR and TS activities were measured at 30 °C under the optimal conditions shown in Figs. 4 and 5. At $t = 0$, trypsin (final concentration, 17 $\mu g\ ml^{-1}$) was added to the reaction medium. Rates are expressed as percent of the initial maximal velocities.

DHPS and DHFS activities in chloroplastic and cytosolic fractions were also observed, but these locations were subject to discussion because of contamination from mitochondria (11). In marked contrast with the results presented here, the DHFR and FPGS activities in mammalian cells were believed to be primarily localized in the cytosol (1, 16, 46). In *Neurospora crassa*, however, ~50% of the FPGS activity was cytosolic and 50% mitochondrial (18). These two FPGS isoenzymes differed in their substrate specificity, the mitochondrial isoform catalyzing glutamate addition only with $H_4PteGlu_2$ as substrate and not with $H_4PteGlu_1$. Thus, it appears from these data that the cell distribution of folate-synthesizing enzymes may greatly vary from one species to another. Yet, it must be pointed out that these localization studies were mainly based on cell fractionation experiments, where inevitable cross-contamination makes the results difficult to interpret. From this point of view, the localization of the enzyme activities in purified cell fractions such as Percoll-purified mitochondria or chloroplasts would permit more definite conclusions. In this context, it has been recently demonstrated that FPGS, with a high specific activity, was also present in mammalian mitochondria purified on a Percoll density gradient (17).

In animal cells, folate is supplied from the external medium, and the mitochondrial pool is obtained from cytosolic folate uptake (47). In plants, the situation is totally different because plant cells can synthesize folate *de novo* within mitochondria. However, tetrahydrofolate derivatives are an absolute requirement for the synthesis of numerous compounds such as purine or thymidylate and must therefore also be present in chloroplasts and cytosol. Thus, taking into account our results, we are forced to imagine that, in marked contrast with the situation

TABLE V

TS activities in the different pea leaf cell compartments and in potato tuber mitochondria

Activities were measured in the presence of CH_2-H_4FGlu_5. The results presented here are the average of five determinations.

	Mitochondria	Chloroplasts	Cytosol	Nuclei	Mitochondria (potato)
	nmol h^{-1} mg^{-1} protein				
TS	7 ± 2	ND[a]	ND	ND	15 ± 3

[a] ND, not detected.

encountered in animal cells, polyglutamyltetrahydrofolates required in chloroplasts and cytosol are, at least partly, supplied from mitochondria. Little is known about folate transport through biological membranes, but the cell membrane permeability to polyglutamylfolate is apparently considerably less than to monoglutamate species (2). Analysis of mitochondrial folate in pea leaves revealed a pool of polyglutamates dominated by tetraglutamate (25%) and pentaglutamate (55%) (32), a situation that therefore will limit the mitochondrial folate ability to be transported across the membranes. Furthermore, tetrahydrofolate polyglutamate is a very labile molecule that undergoes rapid oxidation in the presence of oxygen (48). This oxidative degradation is, however, prevented when tetrahydrofolate is bound to folate-dependent proteins, a situation greatly favored by long polyglutamate chains (48). These observations strongly suggest that tetrahydrofolate cannot diffuse freely within the cell. Unfortunately, there is at present no information available regarding the cellular traffic of folate, and the questions concerning the mode of transport of this cofactor between the different compartments (cotransport with a bind-

ing protein, endocytosis, or potocytosis (49)) remain to be answered.

The specific activities of the enzymes involved in tetrahydrofolate synthesis in plant mitochondria were rather low (1.5–15 nmol h^{-1} mg^{-1} matrix protein), except for DHFR. Indeed, DHFR had a specific activity 10–100 times higher than the other proteins, suggesting a possible role in other metabolic functions. As a matter of fact, DHFR in plant mitochondria is involved not only in tetrahydrofolate synthesis, but also in thymidylate synthesis. This situation is also found in protozoa (43, 50, 51). It is interesting to note that all the experiments done with various species of protozoa (51) indicated only one DHFR/TS activity. The strong analogy between the bifunctional plant enzyme and that from protozoa suggests that, in these latter organisms, DHFR/TS is also localized in mitochondria. In contrast, DHFR in mammalian cells appeared to be a monomeric cytosolic enzyme separated from TS activity (15). Although the bifunctional DHFR/TS enzyme had been intensively studied at the level of molecular biology (13, 52), there is little information concerning its biochemical properties. Our preliminary studies indicate that DHFR/TS has similar properties to monofunctional DHFR. In particular, both DHFRs exhibit a high affinity for either the monoglutamate or the polyglutamate form of dihydrofolate, which illustrates that both types of enzymes play a dual role in tetrahydrofolate biosynthesis and in recycling of H_2FGlu_n formed during thymidylate biosynthesis.

Finally, our results indicate that, in plants, TS was mainly localized in mitochondria. Again, the situation is very different in animal cells, where TS is a monofunctional protein localized in the cytosol and, in the case of actively dividing tissue, closely associated with nuclei (46). Although we cannot rule out the possibility that TS cell distribution in actively dividing plant tissues is different from that reported here, mitochondria from young leaves appeared to be a major site for thymidylate synthesis. This raises the question of the transport of thymidylate toward nuclei and plastids, a point currently under investigation.

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