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Distribution of Folate Derivatives and Enzymes for Synthesis of 10-Formyltetrahydrofolate in Cytosolic and Mitochondrial Fractions of Pea Leaves[1]

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Leaf extracts of 14-d-old pea (*Pisum sativum* L. cv Homesteader) seedlings were examined for folate derivatives and for 10-formyltetrahydrofolate synthetase (SYN), 5,10-methenyltetrahydrofolate cyclohydrolase (CYC), and 5,10-methylenetetrahydrofolate dehydrogenase (DHY) activities. Microbiological and enzyme assays showed that leaf folates SYN, CYC, and DHY were predominantly cytosolic. Extracts of Percoll gradient-purified mitochondria contained less than 1% of total leaf folate and less that 1% of each enzyme activity. Fractionation of whole-leaf homogenates resulted in the copurification of DHY and CYC (subunit 38 kD) and the isolation of a SYN protein (subunit 66 kD). Polyclonal antibodies were raised against purified cytosolic DHY-CYC (DHY-CYC-Ab) and cytosolic SYN (SYN-Ab), respectively. Immunoblots showed that DHY-CYC-Ab cross-reacted with a mitochondrial protein band (38 kD). Two mitochondrial protein bands (subunit M_r = 40,000 and 44,000) cross-reacted with SYN-Ab. Immunoaffinity chromatography (DHY-CYC-Ab as the immobile ligand) indicated that the bulk of mitochondrial SYN activity was not associated with mitochondrial DHY or CYC. When 9-d-old etiolated pea seedlings were exposed to light for up to 3 d, the specific enzyme activities of DHY-CYC in whole-leaf extracts rose 2-fold and more DHY-CYC-Ab cross-reacting protein was detected. In contrast, the specific activity of SYN fell from 5 to 1 μmol min^{-1} mg^{-1} protein and less SYN-Ab cross-reacting protein was detected. The data suggest that in pea leaves, the bulk of one-carbon-substituted tetrahydrofolates and enzymes for the generation of 10-formyltetrahydrofolate are extra-mitochondrial.

Substituted tetrahydrofolates supply one-carbon units for nucleic acid biosynthesis, formation of mitochondrial and chloroplastic proteins, metabolism of Gly, Ser, and His, methyl group biogenesis, and metabolism of some vitamins (Cossins, 1987; Appling, 1991). In eukaryotic cells these reactions are highly compartmented, as evidenced by the association of folates and folate-dependent enzymes with the cytoplasm, mitochondria, and chloroplasts (Cossins, 1980, 1987; Appling, 1991). In plants one-carbon units are mainly generated (Fig. 1) by SYN, SHMT, and GDC (Cossins, 1980). The folate products of these reactions are probably interconvertible, since DHY and CYC are prevalent (Cossins, 1980, 1987; MacKenzie, 1984). This view is also supported by the roles of Ser and formate as precursors of one-carbon units needed for the biosynthesis of purines, thymidylate, and Met (for review, see Cossins, 1987).

The SYN, DHY, and CYC activities of mammalian and yeast cells are catalyzed by C_1-tetrahydrofolate synthase, a trifunctional protein that has cytosolic and mitochondrial forms encoded by separate genes (MacKenzie, 1984; Appling, 1991). In some bacteria these activities are associated with monofunctional proteins (MacKenzie, 1984). In this regard, the *Clostridium thermoacetium* and *Clostridium acidiurici* cDNAs that encode a monofunctional SYN protein have been sequenced (Whitehead and Rabinowitz, 1988; Lovell et al., 1990). In contrast, *Escherichia coli* (Dev and Harvey, 1978; D'Ari and Rabinowitz, 1991) and *C. thermoaceticum* (Ljungdahl et al., 1980) express a bifunctional DHY-CYC complex that lacks SYN activity. The structural organization and compartmentation of these proteins in plants is less certain. In spinach (*Spinacia oleracea* L.) leaves (Nour and Rabinowitz, 1991) and pea (*Pisum sativum* L.) cotyledons (Kirk et al., 1994), SYN activity is associated with a protein that lacks DHY and CYC activities. The SYN protein of pea cotyledons occurs principally as a cytosolic homodimer (subunit M_r = 56,000) and uses $H_4PteGlu_n$ as the preferred folate substrates (Kirk et al., 1994). The spinach leaf SYN has structural homologies to the SYN domains of mammalian and yeast C_1-tetrahydrofolate synthase, as well as a putative sequence for binding polyglutamyl folates (Nour and Rabinowitz, 1992). Further work on pea cotyledon extracts showed that DHY and CYC activities were associated with a bifunctional, homodimeric protein (subunit M_r = 38,500) that was principally cytosolic and that lacked SYN activity (Kirk et al., 1995). It is not clear if these activities have a similar structural organization in leaf tissues.

Leaf mitochondria contain $H_4PteGlu_n$ derivatives (Besson et al., 1993) and folate-dependent enzymes, including SHMT, GDC, dihydrofolate reductase, and thymidylate

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Abbreviations: 5,10-CH_2-H_4PteGlu, 5,10-methylenetetrahydropteroylmonoglutamate; DHY-CYC, 5,10-methylenetetrahydrofolate dehydrogenase:5,10-methenyltetrahydrofolate cyclohydrolase bifunctional protein; DHY-CYC-Ab and SYN-Ab, polyclonal antibodies raised to the cytosolic forms of DHY-CYC and SYN, respectively; GDC, Gly decarboxylase; 10-HCO-H_4PteGlu, 10-formyltetrahydropteroylmonoglutamate; $H_4PteGlu_n$, tetrahydropteroylpolyglutamate derivatives; SHMT, Ser hydroxymethyltransferase; SYN, 10-formyltetrahydrofolate synthetase.

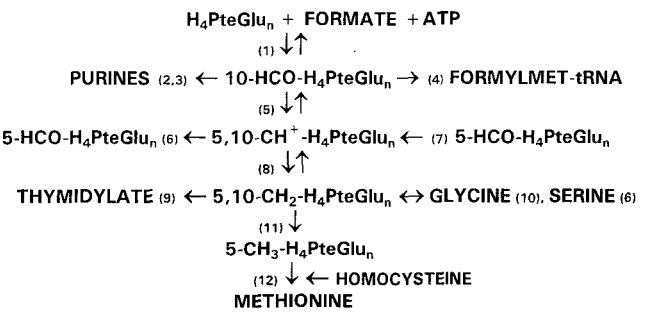

Figure 1. Major folate-dependent pathways of one-carbon metabolism. Enzymes catalyzing individual reactions are: (1) SYN (EC 6.3.4.3); (2) phosphoribosylglycinamide formyltransferase (EC 2.1.2.2); (3) phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3); (4) methionyl-tRNA formyltransferase (EC 2.1.2.9); (5) 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9); (6) Ser hydroxymethyltransferase (EC 2.1.2.1); (7) 5,10-methenyltetrahydrofolate synthetase, (EC 6.3.3.2); (8) 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5); (9) thymidylate synthase (EC 2.1.1.45); (10) Gly cleavage complex (EC 1.4.4.2/2.1.2.10); (11) 5,10-methylenetetrahydrofolate reductase, (EC 1.5.1.20); and (12) Met synthase, (EC 2.1.1.14).

synthase (Neuburger et al., 1996). In addition, these mitochondria contain key enzymes of a folate biosynthetic pathway that conceivably forms $H_4PteGlu_n$ from pteridine precursors (Neuburger et al., 1996). Although it is not clear if this pathway generates all of the $H_4PteGlu_n$ needed to support cellular one-carbon metabolism, there is good evidence that the mitochondria may be the major site for dihydropteroate biosynthesis in plant cells (Rebeille et al., 1997).

Other studies have clearly shown that the total folate pool is enlarged when etiolated leaves are exposed to light (Spronk and Cossins, 1972). Light also increases the specific activities of SYN, DHY (Gifford and Cossins, 1982), SHMT, and GDC (Walker and Oliver, 1986; Turner et al., 1993; Oliver and Raman, 1995). Greening in the presence of α-hydroxy-2-pyridinemethane sulfonate reduced this enhancement of SYN activity in barley leaves, as well as the ability to metabolize labeled formate. Thus, formate for the SYN reaction in barley leaves may be partly derived from glycollate during photorespiration (Gifford and Cossins, 1982). Conceivably, leaf mitochondria, like those of nongreen tissues (Cossins, 1980, 1987), also contain enzymes that oxidize part of the $5,10\text{-}CH_2\text{-}H_4PteGlu_n$ arising from the photorespiratory cleavage of Gly, but there is still little information on this aspect of plant folate metabolism. The present work has therefore examined the distribution of folate derivatives and SYN, DHY, and CYC activities in cytosolic and mitochondrial fractions of pea leaves.

MATERIALS AND METHODS

Reagents

Folic acid, (6*R*,*S*)-H_4PteGlu, heparin agarose, and Sephacryl S-200 and S-300 were supplied by Sigma. Matrex Green A and Matrex Orange A were purchased from Amicon (Beverly, MA). Bio-Gel P-6, hydroxyapatite, and Affi-Gel Hz Hydrazide Gel were obtained from Bio-Rad. DEAE-52 cellulose was obtained from Whatman. Chemiluminescence western-blot kits were supplied by Boehringer Mannheim and other reagents for immunoblot assays were from Sigma. The HPLC of folates used a μBondapak C_{18} reverse-phase column supplied by Waters. *Lactobacillus rhamnosus* (ATCC 7469) was obtained from the American Type Culture Collection (Rockville, MD), and microbiological media were from Difco Laboratories (Detroit, MI). All other reagents were purchased from Sigma or Fisher Scientific.

Plant Material

Pea (*Pisum sativum* L. cv Homesteader) seedlings were grown under controlled conditions (Imeson et al., 1990) with leaves harvested at 14 d. The leaves of etiolated seedlings, grown for 9 d under these conditions but in darkness, were harvested in subdued light (15-W florescent bulb, Green no. 7, Kodak filter). Extracts were prepared immediately after excision.

Microbiological Assay of Folates

Whole-leaf extracts were prepared for folate analysis as described earlier (Spronk and Cossins, 1972). Cytosolic and mitochondrial fractions were isolated by the method of Douce et al. (1987). The cytosolic fraction was diluted 3-fold by the addition of either ascorbate (Clandinin and Cossins, 1972) or Ches buffer containing ascorbate and 2-mercaptoethanol (Wilson and Horne, 1984). Mitochondria were resuspended in these buffers containing 0.1% Triton X-100 and held at 4°C for 1 h. These cytosolic and mitochondrial extracts were incubated at 100°C for 10 min and centrifuged, and the supernatants were retained for folate analysis. Polyglutamyl folates were hydrolyzed using pea cotyledon γ-glutamyl hydrolase (Lin et al., 1993). Reaction systems contained 5% (w/v) sodium ascorbate and 75 mM acetate buffer (pH 6.0). Folate analyses were carried out using cryoprotected cells of *L. rhamnosus* (Wilson and Horne, 1982) and microtiter plates (Horne and Patterson, 1988). A_{630} was read using a Microplate Autoreader (model EL311, Bio-Tek Instruments, Inc., Winooski, UT) interfaced to a computer. Samples of these extracts were subjected to HPLC on a μBondapak C_{18} reverse-phase column to separate individual folate derivatives (Horne et al., 1981, 1989).

Enzyme Assays

SYN, DHY, and CYC were assayed spectrophotometrically using monoglutamyl folate substrates and the reaction conditions of Kirk et al. (1995). Alcohol and succinate dehydrogenase were assayed as marker enzymes of the cytosolic and mitochondrial fractions, respectively (Coffin and Cossins, 1986). Protein was measured by the method of Bradford (1976).

Organelle Isolation and Preparation of Enzyme Extracts

The preparation of leaf extracts and the isolation of purified mitochondria were done according to Douce et al. (1987). The 12,000*g* supernatant fraction was used as a source of cytosolic proteins. Chloroplasts were isolated according to Schuler and Zielinski (1989). Mitochondria and chloroplasts were resuspended in 25 mM Hepes buffer (pH 7.5) containing 0.1% Triton X-100, 1 mM PMSF, 10 mM 2-mercaptoethanol, 10 mM KCl, and 20% glycerol (v/v). These suspensions were sonicated (two pulses of 1.5 min at 4°C) using an ultrasonic homogenizer (series 4710, Cole-Parmer Instrument Co., Chicago, IL) with the output control set at 70. The resulting extracts were used in enzyme assays, immunoblots, and for immunoaffinity chromatography.

Purification of Cytosolic DHY-CYC

The aerial shoots of 14-d-old seedlings were homogenized in 50 mM Tris-HCl (pH 7.5) containing 25% (v/v) glycerol, 10 mM 2-mercaptoethanol, and 1 mM PMSF (buffer A). The homogenate was centrifuged, treated with streptomycin sulfate, $(NH_4)_2SO_4$, and chromatographed on heparin agarose (Kirk et al., 1995) to yield step-4 protein. After concentrating by ultrafiltration (Centriprep-10, Amicon) and desalting (Bio-Gel P-6) these extracts were chromatographed on Matrex Green A (Kirk et al., 1995) to give step-5 protein. Enzyme-active fractions were pooled, concentrated as before, and applied to a 3- × 80-cm column of Sephacryl S-200. Protein was eluted with buffer A containing 10 mM Tris-HCl. Fractions containing DHY-CYC were combined, concentrated (see above), and applied to a 1.5- × 6-cm column of Matrex Orange A pre-equilibrated in buffer A containing 10 mM Tris-HCl. The column was washed with the loading buffer (25 mL), and DHY-CYC was eluted with a linear KCl gradient (0.05–0.4 M in the loading buffer).

Purification of Cytosolic SYN

Homogenate preparation and $(NH_4)_2SO_4$ fractionation were done according to Nour and Rabinowitz (1991). The 50 to 70% $(NH_4)_2SO_4$ fraction was dissolved in 25 mM Hepes buffer (pH 7.5) containing 1 mM PMSF, 10 mM 2-mercaptoethanol, 10 mM KCl, and 20% (v/v) glycerol (buffer B). The extract was desalted using Bio-Gel P-6 columns and protein was applied to a 5- × 20-cm column of DEAE-52 cellulose pre-equilibrated with buffer B. The column was washed with 2.5 to 3 L of buffer B until A_{280} of the wash was negligible. SYN activity was eluted using a KCl linear gradient (0.01–0.3 M) in the loading buffer to give step-3 protein. The combined SYN fractions were concentrated as above and diluted in buffer B to give a final KCl concentration of 50 mM before loading onto a heparin agarose column (2.5 × 6 cm) pre-equilibrated with buffer B containing 50 mM KCl. The column was washed with 200 mL of the pre-equilibration buffer, and SYN was eluted with a linear gradient of 50 to 500 mM KCl in 400 mL of the loading buffer. SYN fractions were pooled, concentrated by ultrafiltration, and chromatographed on hydroxyapatite as described by Nour and Rabinowitz (1991).

Polyclonal Antibodies, ELISA, and Western-Blot Analyses

Samples of purified DHY-CYC and SYN were lyophilized and used for the preparation of polyclonal antibodies in rabbits. For the first injection, about 85 μg of the purified proteins was dissolved in 0.8 mL of 0.8% (w/v) sterile saline and homogenized to emulsion with 0.8 mL of Freund's complete adjuvant. After 1 month an injection of 45 μg of protein in Freund's incomplete adjuvant was administered. One month later a test bleed was done to check the titer of antisera. If this was satisfactory, the rabbits received a final injection of 45 μg of protein in Freund's incomplete adjuvant; serum was collected after 1 week. IgG was partially purified by Na_2SO_4 precipitation and DEAE-cellulose column chromatography (Johnstone and Thorpe, 1987) before lyophilization and storage at −20°C. For ELISA, microtiter plates were coated with purified DHY-CYC or SYN, washed with PBS buffer (pH 7.5), blocked with 1% BSA, and incubated with dilutions of antisera. The wells were then washed with PBS buffer and incubated with a goat anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase. A_{405} was measured after the addition of *p*-nitrophenyl phosphate. SDS-PAGE and western-blot transfers employed a Mini Protein II system (Bio-Rad) with electrophoresis on 4% stacking and 12% separating SDS-PAGE slab gels, according to the manufacturer. Protein samples were denatured by mixing with 4 volumes of sample buffer (0.25 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.00125% bromphenol blue) and boiling for 5 min prior to SDS-PAGE. Protein bands were detected by silver-staining (Bio-Rad instructional manual). DHY-CYC-Ab and SYN-Ab were primary antibodies in western blots. Cross-reacting proteins were detected by chemiluminescence (Boehringer Mannheim).

Immunoaffinity Chromatography of Mitochondrial Extracts

Twenty milligrams of partially purified DHY-CYC-Ab was coupled (approximately 60% efficiency) to 10 mL of Bio-Rad Affi-Gel Hz Hydrazide Gel, according to the manufacturer. Five milliliters of the gel, in a Bio-Rad Econo column, was pre-equilibrated with buffer B and mitochondrial extract was applied at room temperature. Unbound protein was collected and assayed for enzyme activity. The column was washed with 15 mL of 1 M GuHCl followed by 30 mL of 0.2 M Gly-HCl buffer (pH 2.8). The collected fractions (1 mL) were immediately neutralized.

RESULTS

Intracellular Distribution of Folates in Pea Leaf Extracts

Initially, we examined the folate content of whole-leaf extracts that were prepared in the absence of an osmoticum. Microbiological assays were carried out after treatment of these extracts with folate hydrolase. This enzyme

Table I. *The intracellular distribution of folate derivatives*

Leaves of 14-d-old seedlings were extracted under isotonic conditions, fractionated by differential centrifugation, and assayed for total folates using *L. rhamnosus* (see "Materials and Methods"). The distribution of individual folate derivatives was determined after HPLC and microbiological assay of column effluents. For folate content, ±SE; $n = 4$.

Fraction	Total Folate Content		Distribution of Major H_4PteGlu derivatives			
			10-Formyl	5-Formyl	Unsubstituted	5-Methyl
	nmol 100 g^{-1} fresh wt	*nmol mg^{-1} protein*	%			
Homogenate[a]	143.4 ± 6.6	0.13 ± 0.01	17.1	18.9	16.8	47.2
1,500*g* Supernatant[b]	111.3 ± 6.6	0.13 ± 0.01	N.A.[c]	N.A.	N.A.	N.A.
12,000*g* Supernatant	93.5 ± 7.6	0.11 ± 0.01	27.0	20.3	N.D.[d]	52.8
Purified mitochondria	0.5 ± 0.1	0.33 ± 0.10	5.8	50.4	37.9	6.0

[a] Initial homogenate was filtered, incubated at 100°C, and centrifuged to remove denatured protein. [b] The pellet from this step contained chloroplasts, nuclei, intact cells, and detectable levels of *L. rhamnosus*-active folates, which were not quantified. [c] N.A., Not assayed. [d] N.D., Not detected.

hydrolyzes long-chained folylpolyglutamates to folylmonoglutamates, which are readily quantified in the *L. rhamnosus* assay (Cossins, 1984). In agreement with earlier work (Spronk and Cossins, 1972), total folate contents (nmol g^{-1} fresh weight) were 4.90 ± 0.54 SE, $n = 3$. HPLC analyses showed that 5-CH_3-H_4PteGlu, a major component of the whole-leaf folate pool, was accompanied by smaller quantities of 10-HCO-, 5-HCO-H_4PteGlu, and H_4PteGlu (data not shown). When extracts were prepared in the presence of an osmoticum (Table I) to facilitate isolation of intact mitochondria (Douce et al., 1987), the less rigorous, grinding procedure resulted in the recovery of less total folate (1.43 ± 0.07 nmol g^{-1} fresh weight). Centrifugation of these extracts showed that folate derivatives were principally associated with the 12,000*g* or cytosolic fraction (Table I). This folate pool was mainly 5-CH_3-H_4PteGlu. The mitochondrial fraction contained much smaller amounts of folate and, in agreement with an earlier study (Besson et al., 1993), these were highly variable between individual extracts. HPLC analyses showed that the mitochondrial folate pool was principally 5-HCO-H_4PteGlu and unsubstituted tetrahydrofolate.

Synthetase, Dehydrogenase, and Cyclohydrolase Activities Are Mainly Cytosolic

SYN, DHY, and CYC activities were readily detected when isotonic extracts were centrifuged to recover cytosolic and mitochondrial fractions. The bulk of these activities were cytosolic (Table II). The small amounts of mitochondrial activity were only detected when extracts were sonicated in the presence of Triton X-100. When these treatments were applied individually, activity was not detected in the mitochondrial fraction. Attempts to detect SYN, DHY, or CYC activities in Percoll gradient-purified chloroplast extracts were not successful, and proteins in these extracts failed to cross-react with DHY-CYC-Ab or SYN-Ab in western-blot analyses (data not shown).

Copurification of Cytosolic DHY and CYC Activities

When PMSF-containing extracts of 14-d-old pea leaves were fractionated (Table III), the ratio of DHY and CYC activities remained relatively constant, indicating a copurification through steps 1 to 7 of the protocol. By step 7, the

Table II. *Distribution of SYN, DHY, and CYC in cytosolic and mitochondrial extracts*

Enzyme Activity	Cytosolic (12,000*g* Supernatant)	Mitochondria Pellet Sonicated + Triton X-100
SYN		
Total units[a]	62.5	0.32
Percent of cytosolic activity	100	0.52
DHY		
Total units[a]	45.4	0.19
Percent of cytosolic activity	100	0.43
CYC		
Total units[a]	25.8	0.14
Percent of cytosolic activity	100	0.54
Alcohol dehydrogenase		
Total units[a]	41.3	n.d.[b]
Percent of cytosolic activity	100	n.d.
Succinate dehydrogenase		
Total units[a]	>0.01	1.01
Percent of cytosolic activity	n.a.[c]	n.a.

[a] Expressed in μmol min^{-1}. [b] n.d., Not detected. [c] n.a., Not applicable.

Table III. *Copurification of DHY and CYC activities during fractionation of whole-leaf extracts*

Fractionation Step	Protein	DHY Activity		CYC Activity		Ratio (DHY/CYC)
		Total	Specific	Total	Specific	
	mg	*μmol min⁻¹*	*μmol min⁻¹ mg⁻¹*	*μmol min⁻¹*	*μmol min⁻¹ mg⁻¹*	
1. Crude homogenate	1880	16.90	0.01	6.34	0.003	2.66
2. Streptomycin SO_4	1143	16.40	0.01	6.05	0.005	2.71
3. 55 to 75% $(NH_4)_2SO_4$	246	6.94	0.03	2.85	0.012	2.43
4. Heparin agarose	37.5	9.94	0.26	3.32	0.090	2.99
5. Matrex Green A	6.0	5.76	0.96	2.41	0.402	2.39
6. Sephacryl S-200	2.5	4.95	1.96	2.09	0.840	2.37
7. Matrex Orange A	0.038	1.32	34.7	0.57	15.00	2.33

purifications achieved were about 3900-fold for DHY activity and 4400-fold for CYC activity; recovery of these activities was 8 to 9%. Considering the very low levels of both activities in whole-mitochondrial extracts (Table II), it follows that step-7 protein was mainly cytosolic. Step-7 protein lacked SYN activity (data not shown), and SDS-PAGE revealed a single, silver-stained protein band with an average M_r of 38,000 (Fig. 2A). Sephacryl S-200 chromatography in the presence of molecular weight markers proteins showed that the DHY-CYC fractions had an apparent molecular mass of about 58 kD. These observations imply that both activities are associated with a bifunctional protein that may be homodimeric.

Purification of Cytosolic SYN

Purification of monofunctional SYN proteins has been reported for spinach leaves (Nour and Rabinowitz, 1991) and pea cotyledons (Kirk et al., 1994). In the present work we used an improved protocol that gave homogeneous SYN protein after chromatography on DEAE-52 cellulose, heparin agarose, and hydroxyapatite (Table IV). By step 5, SYN was purified about 650-fold with a recovery of about 17%. The level of activity recovered, compared with that found in whole-mitochondrial extracts (Table II), suggests that step-5 protein was mainly derived from the cytosolic form of this enzyme. SDS-PAGE revealed a single, silver-stained protein band with an average molecular mass of 66 kD (Fig. 2B). This protein lacked DHY and CYC activities. Gel filtration on Sephacryl S-300 indicated an average value of 120 kD. Thus, the SYN of pea leaves, like that of spinach leaves and pea cotyledons, appears to be homodimeric.

Immunoblot and Immunoaffinity Chromatography of Mitochondrial Extracts

Polyclonal antibodies were raised in rabbits against step-7 DHY-CYC cytosolic protein and against step-5 SYN cytosolic protein. The titer of both polyclonals is shown in Figure 3. IgG was partially purified and lyophilized prior to use in immunological studies. It is clear that DHY-CYC-Ab cross-reacted with a single band (molecular mass of 38 kD) in whole-leaf and -mitochondrial extracts (Fig. 4A). The mobility of these bands was like that of purified cytosolic DHY-CYC (Fig. 2A). An immunoaffinity column, containing bound DHY-CYC-Ab, was used in an attempt to fractionate the low levels of SYN, DHY, and CYC activity in whole-mitochondrial extracts.

When DHY-CYC-Ab was used as an immobile ligand, 90% of the DHY and CYC activities in whole-mitochondrial extracts was retained by the column, whereas approximately 90% of the SYN activity passed through the column and was recovered in the column wash (Table V). This suggests that the bulk of mitochondrial SYN is not associated, as in yeast and mammalian cells, with DHY and CYC activities. Although enzyme activities could not be detected in the neutralized Gly-HCl buffer eluant, several silver-stained protein bands were recovered (Fig. 5A). Immunoblots of the column wash (Fig. 5B, lane 2) and column eluant (Fig. 5B, lane 3) showed that some of these proteins cross-reacted with the goat anti-rabbit secondary antibody (Fig. 5B). This indicates that some loosely coupled rabbit IgG was released from the column matrix. Despite this release, an additional cross-reacting band of approximately 38 kD was only revealed when DHY-CYC-Ab was used as the primary antibody (Fig. 5, B and C).

Western blots using SYN-Ab as the primary antibody (Fig. 4B) revealed two bands (44 and 40 kD) in mitochondrial extracts, one band (66 kD) in purified SYN, and four major bands (63, 45.5, 44, and 28 kD) in whole-leaf extracts.

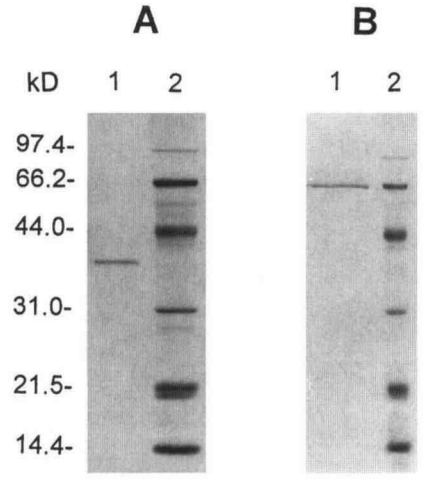

Figure 2. SDS-PAGE of cytosolic DHY-CYC protein (A) and SYN protein (B). A, DHY-CYC protein after Matrex Orange A purification (lane 1), molecular weight standards (lane 2). B, SYN after hydroxyapatite chromatography (lane 1), molecular weight standards (lane 2). Protein bands were detected by silver-staining.

Table IV. *Purification of SYN activity from whole-leaf extracts*

Fractionation Step	Protein	Activity		Purification	Recovery
		Total	Specific		
	mg	*μmol min⁻¹*	*μmol min⁻¹ mg⁻¹*	*fold*	%
1. Initial extract	2259	12.29	0.005	1	100
2. 50–70% $(NH_4)_2SO_4$	722	7.03	0.010	2	57.2
3. DEAE-52 cellulose	8.04	5.34	0.664	123	43.4
4. Heparin agarose	1.45	3.12	2.155	400	25.4
5. Hydroxyapatite	0.58	2.04	3.565	650	16.6

The Effect of Greening on the Expression of DHY, CYC, and SYN

When 9-d-old etiolated pea seedlings were exposed to light for a period of 72 h, the specific activity of SYN in whole-leaf extracts dropped 4-fold, whereas the specific enzyme activities of DHY and CYC rose about 2-fold (Fig. 6). Immunoblots suggested that greening also affected the amounts of cross-reacting protein (Figs. 7 and 8). Thus, the amount of SYN-Ab-reacting protein, as judged by western-blot analyses, decreased dramatically in extracts of plants exposed to light (Fig. 7). A smaller, but significant, decrease in SYN-Ab-reacting 66-kD protein also occurred in the plants maintained in the dark (Fig. 7). Thus, the levels of this protein may decline during seedling development. In contrast, the amount of DHY-CYC-Ab cross-reacting protein increased during greening (Fig. 8A). The decreases noted for SYN-Ab-reacting protein appeared to include the 44-kD band associated with mitochondrial extracts (Fig. 4B). Assay of mitochondrial extracts, prepared from etiolated and greening seedlings (Table VI), showed that the specific activity of SYN decreased by more than 2-fold following 48 h of illumination, but the levels of DHY and CYC were not appreciably changed. Immunoblots of these mitochondrial extracts suggested that the amounts of DHY-CYC cross-reacting protein remained approximately the same before and after the light treatment (Fig. 8B).

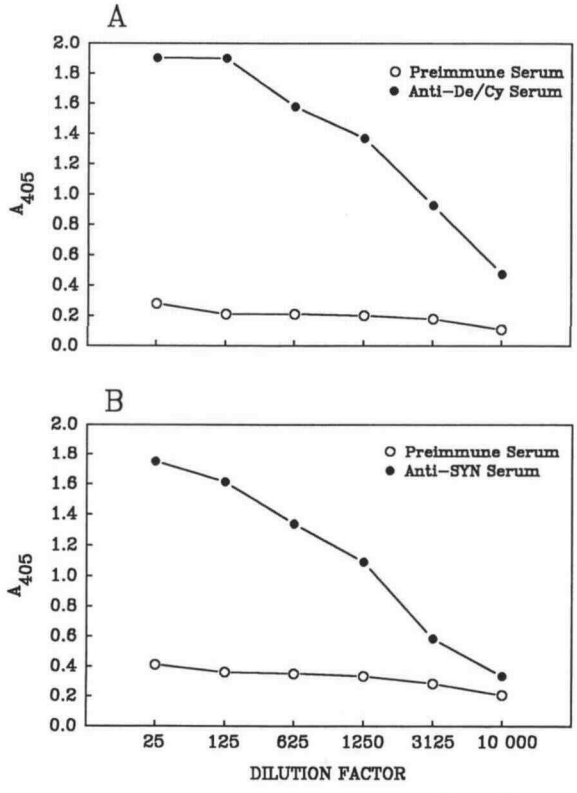

Figure 3. Reactivity of antisera against DHY-CYC (De/Cy) (A) and SYN (B) proteins. ELISA measurements (see "Materials and Methods") used microtiter wells coated with purified DHY-CYC or SYN protein containing dilutions of anti- or preimmune sera. Secondary antibody was goat anti-rabbit IgG-conjugated to alkaline phosphatase (Sigma), and each value is the mean of triplicate determinations. The SE was less than 5%.

DISCUSSION

Earlier studies (Imeson et al., 1990) established that the folate pool of pea leaves is highly glutamyl-conjugated. Table I shows that most of this folate is cytosolic, and in this compartment 5-CH_3-H_4PteGlu$_n$ accounts for more than 50% of the recovered folate. The cytosolic pool also contains 10-HCO-H_4PteGlu$_n$. Since the bulk of SYN, DHY, and CYC activities occur in the cytosol (Table II), it follows that this compartment may be a major site for the interconversion of 10-HCO-H_4PteGlu$_n$ and 5,10-CH_2-H_4PteGlu$_n$. In

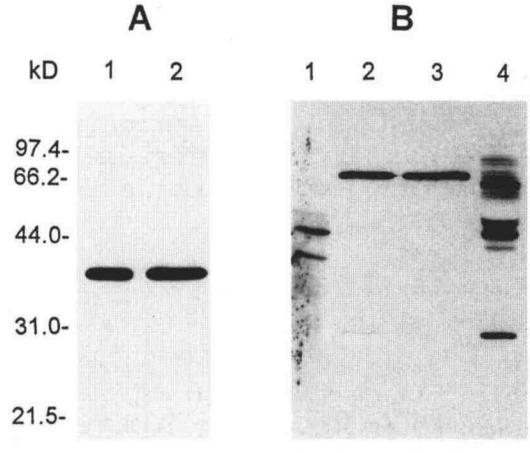

Figure 4. Immunoblots of whole-leaf and -mitochondrial extracts of 14-d-old seedlings using DHY-CYC-Ab (A) and SYN-Ab (B). A, Whole-leaf extract, 20 μg of protein (lane 1); and extract of gradient-purified mitochondria, 30 μg of protein (lane 2). B, Gradient-purified mitochondria, 25 μg of protein (lane 1); purified cytosolic SYN, 40 and 50 ng of protein (lanes 2 and 3, respectively); and whole-leaf extract, 25 μg of protein (lane 4).

Table V. *Immunoaffinity chromatography of mitochondrial SYN, DHY, and CYC activities*

Total activities (μmol min^{-1}), present in the initial mitochondrial extract, were used to calculate the percentages of each activity recovered in the column fractions. After application of the mitochondrial extract, the affinity column, containing DYH/CYC-Ab as an immobile ligand, was washed with the loading buffer to obtain "unbound" fractions followed by washing with GuHCl and 0.2 M Gly-HCl (pH 2.8) to obtain "eluant" fractions (see "Materials and Methods").

Fractions Recovered	SYN	DHY	CYC
		%	
Initial extract	100	100	100
Unbound fractions	90	8.3	9.4
Eluant fractions	n.d.[a]	n.d.	n.d.

[a] n.d., Activity not detectable by the standard enzyme assay procedures.

a recent study Neuburger et al. (1996) used a radioassay procedure to examine the intracellular distribution of folate in pea leaves. These workers detected 0.40 ± 0.05 nmol folate mg^{-1} protein in purified mitochondria, a value similar to that shown in Table I. They concluded that the mitochondrial compartment accounted for most of the cellular folate. In contrast, we found that less than 1% of leaf folates was associated with the mitochondrial fraction (Table I). The reason for this discrepancy may include significant losses of cytosolic folate during extract concentration, and the fact that radioassays are generally less sensitive than microbiological assays (Cossins, 1984). It has also been reported that radioassays have limited value in the measurement of folylpolyglutamates (Shane et al., 1980). If all cellular folates are synthesized de novo by the mitochondria (Neuburger et al., 1996), it follows that precursors of the larger, cytosolic folate pool must be transported across mitochondrial membranes. This possibility warrants further study.

Table I shows that the mitochondrial folate pool contains principally 5-HCO-H_4PteGlu$_n$ and H_4PteGlu$_n$. The latter derivative is a major component of rat liver mitochondria (Horne et al., 1989; Balaghi et al., 1993), where it participates in several pathways of folate-mediated metabolism (Appling, 1991). It follows that in leaf mitochondria H_4PteGlu$_n$ would have importance in the interconversion of Gly and Ser (Cossins and Chen, 1997), but the origins and metabolic roles of 5-HCO-H_4PteGlu$_n$ are not clear. In animals this folate is derived from 5,10-methenyltetrahydropteroylpolyglutamate in a reaction catalyzed by SHMT (Stover and Schirch, 1990). The inhibition of SHMT by 5-HCO-H_4PteGlu$_n$ also appears to regulate the production of one-carbon units in mammals (Stover and Schirch, 1991). The role of this folate in plant mitochondria should therefore be elucidated.

Our earlier studies of pea cotyledon extracts showed that the SYN and DHY activities were predominately cytosolic, with only minimal activities being recovered in mitochondria of 6- and 7-d-old seedlings (Kirk et al., 1994, 1995). These tissues also have a cytosolic CYC activity, but a comparable mitochondrial enzyme was not detected in sonicated, detergent-treated extracts. It is clear from Table II that all three activities occur in the cytosolic and mitochondrial compartments of pea leaves. The requirement for sonication and detergent treatments to reveal activity in the mitochondria suggests that these activities are latent and are possibly associated with the inner mitochondrial membrane. It is surprising that we could not detect SYN, DHY, or CYC in the chloroplast fraction by enzyme assay or by western-blot analyses. Like mitochondria, these organelles have a pool of 10-HCO-H_4PteGlu (Cossins and Shah, 1972) and they utilize this folate in the transformylation of methionyl-tRNA (Staben and Rabinowitz, 1984; Cossins, 1987). Furthermore, isolated pea chloroplasts convert formate to Ser (Shingles et al., 1984) and have SHMT activity

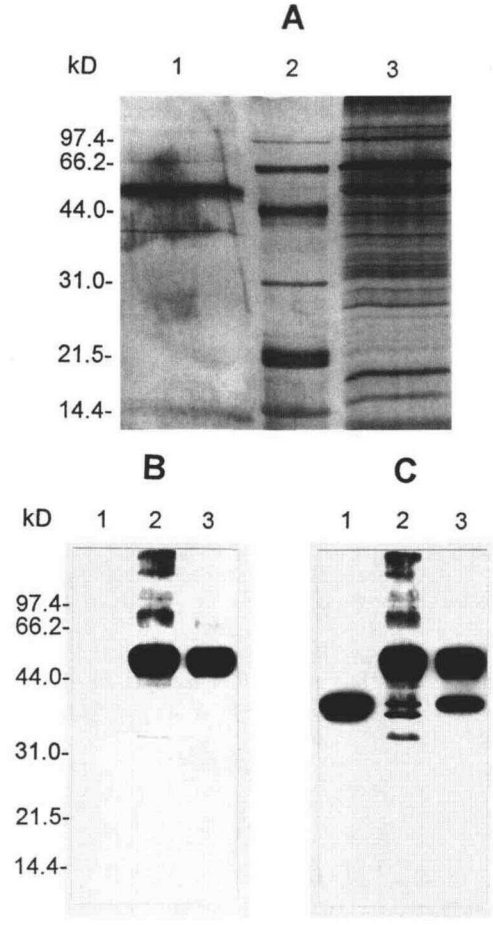

Figure 5. SDS-PAGE (A) and immunoblots (B and C) of mitochondrial proteins before and after immunoaffinity chromatography. The polyclonal antibodies (DHY-CYC-Ab) were raised against purified cytosolic DHY-CYC protein (see "Materials and Methods"). A, Mitochondrial proteins that were bound and then eluted from the immunoaffinity column (lane 1); molecular weight standards (lane 2); and whole-mitochondrial extract before chromatography (lane 3). B, Western blots in which the primary antibody incubation step with DHY-CYC-Ab was omitted. C, Western blots using DHY-CYC-Ab as the primary antibody. Whole-mitochondrial extract before immunoaffinity chromatography (lane 1); mitochondrial proteins that passed through the column and were collected in the initial column wash (lane 2); and proteins that were retained by the column and then eluted with Gly-HCl buffer, pH 2.8 (lane 3).

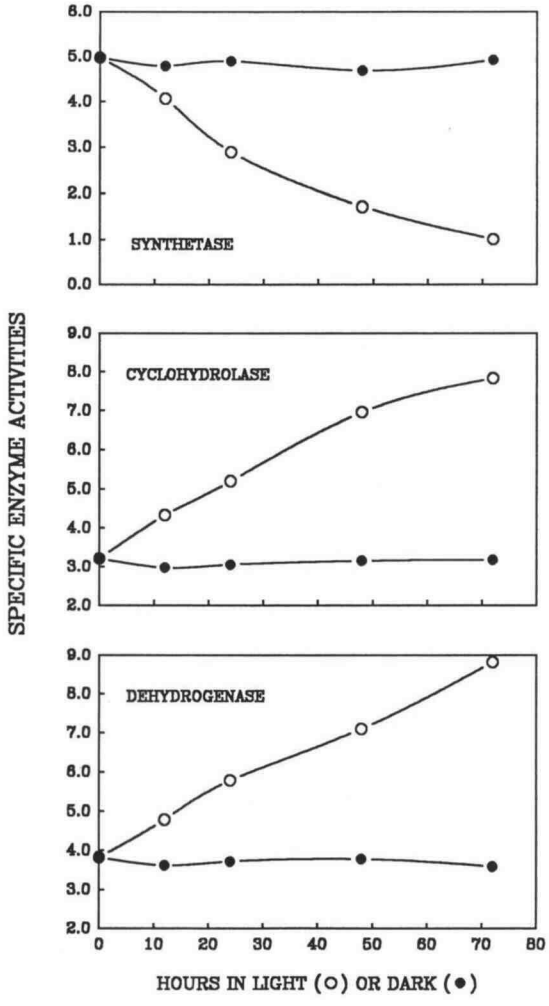

Figure 6. The effect of greening on specific enzyme activities. Nine-day-old, dark-grown plants were exposed to light (open symbols) for the periods indicated or maintained in the dark throughout the experiment (closed symbols, controls). Whole-leaf extracts were assayed. Each point is the mean of three separate determinations with SEs between 8 and 13%. Specific activities are expressed in nmol min^{-1} mg^{-1}.

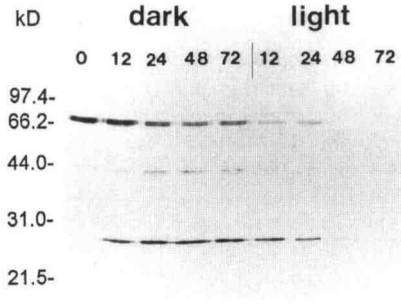

Figure 7. Western blots of SYN protein in extracts of light-treated etiolated pea leaves. The numbers above each lane indicate hours of light or dark exposure of seedlings that were initially grown in darkness for 9 d. Twenty micrograms of protein was loaded in each lane.

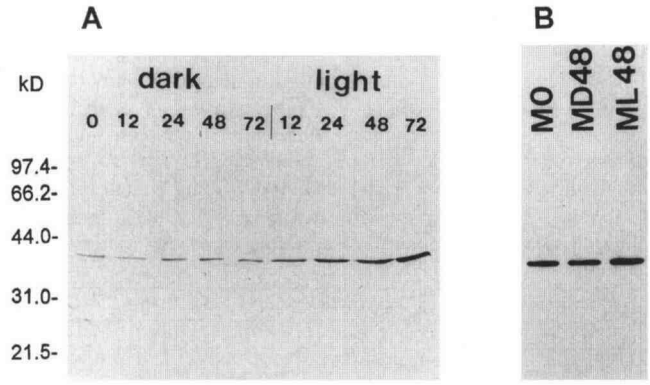

Figure 8. Western blots of DHY-CYC protein in extracts of light-treated etiolated leaves. A, Whole-leaf extracts; 20 μg of protein loaded per lane. B, Mitochondrial extracts of greening leaves; 5 μg of protein loaded per lane. The numbers above each lane indicate hours of light or dark exposure of seedlings initially grown in darkness for 9 d. MO, Mitochondrial extract prepared from leaves of plants grown in darkness for 9 d. Leaf mitochondrial extracts of 9 d and, dark-grown plants that had been maintained in darkness for an additional 48 h (MD48), or placed in the light for 48 h (ML48).

(see Cossins, 1980). Thus, pea chloroplasts should also have the enzymes to generate formyl-, methenyl- and methylenetetrahydrofolates. In this regard, Neuburger et al. (1996) recently detected DHY activity in isolated pea chloroplasts. Our failure to detect these proteins in chloroplast extracts suggests that they may be structurally and catalytically distinct from their cytosolic counterparts.

When leaf extracts were fractionated in the presence of PMSF (Tables III and IV), the DHY and CYC activities were clearly separated from SYN activity. Based on SDS-PAGE and silver-staining, DHY-CYC and SYN were purified to apparent homogeneity (Fig. 2). The levels of recovered enzyme activity suggest that both purified proteins were mainly of cytosolic origin. The copurification of DHY and CYC (Table III) indicates an association of these activities, as reported for pea cotyledons (Kirk et al., 1995). On the other hand, a separate SYN protein, which lacked DHY and CYC, was isolated from leaf extracts (Table IV). The purified SYN protein had a subunit M_r of 66,000 and is therefore similar to that reported from spinach leaf extracts (Nour and Rabinowitz, 1991). Cloning and sequencing of the spinach gene encoding this protein (Nour and Rabinowitz, 1992) indicated homologies with the SYN domain of the mammalian and yeast trifunctional C_1-tetrahydrofolate synthases. These authors concluded that spinach SYN is catalyzed by a monofunctional protein. The present work suggests that a similar protein is expressed in the cytosolic compartment of pea leaves.

Attempts to purify these enzymes from mitochondrial extracts were not successful because of their low levels and relatively poor stability. However, western-blot analyses (Fig. 4) showed that DHY-CYC-Ab detected a mitochondrial cross-reacting protein of approximately the same subunit size as the purified, cytosolic DHY-CYC. In addition, most of the mitochondrial DHY and CYC activities were retained when extracts were passed through an immunoaffinity column containing bound DHY-CYC-Ab (Table V).

Table VI. *The effect of greening on the specific activities of mitochondrial SYN, DHY, and CYC*

Seedlings were grown for 9 d in darkness and then transferred to continuous light for 48 h. Controls were maintained in the dark for a total period of 11 d. Mitochondria were isolated, purified, and suspended in 25 mM Hepes buffer (pH 7.5), containing 0.1% Triton X-100, 1 mM PMSF, 10 mM 2-mercaptoethanol, 10 mM KCl, and 20% glycerol and assayed for each enzyme activity (see "Materials and Methods"). Data are the averages of three separate determinations (± SE).

Mitochondrial Extracts	SYN	DHY	CYC
		nmol min^{-1} mg^{-1}	
Dark grown, 9 d	3.73 ± 0.41	3.54 ± 0.29	4.48 ± 0.56
Dark grown, 11 d	3.93 ± 0.35	3.36 ± 0.35	4.62 ± 0.60
Light grown, 48 h[a]	1.11 ± 0.12	2.89 ± 0.24	4.07 ± 0.43

[a] Nine-day-old etiolated plants were transferred to light for 48 h.

Thus, it appears likely that pea leaf mitochondria have DHY and CYC activities that are associated as in the cytosolic protein. This association does not appear to include SYN, since the bulk of this mitochondrial activity passed through the immunoaffinity column (Table V) and SYN-Ab did not cross-react with mitochondrial protein of 38 kD (Fig. 4). In these respects the structural organization of DHY, CYC, and SYN in pea leaf mitochondria appear to be distinct from that reported for yeast and mammalian mitochondria where all three activities are associated with a protein of a subunit mass of 100 kD (Shannon and Rabinowitz, 1986; Appling, 1991).

The monofunctional SYN proteins characterized to date exhibit homology with the synthetase domain of C_1-tetrahydrofolate synthases and have a subunit mass of approximately 60 kD (Whitehead and Rabinowitz, 1988; Lovell et al., 1990; Nour and Rabinowitz, 1992). It is therefore surprising that our SYN-Ab, raised against the purified cytosolic SYN, only cross-reacted with mitochondrial proteins of 40 and 44 kD (Fig. 4B). Failure to detect mitochondrial protein of approximately 60 kD was not due to insufficient antibody titer, as these SYN-Ab preparations readily detected comparable low levels of cytosolic SYN (data not shown). Plausible explanations for these observations might include the following. First, pea mitochondrial SYN is not structurally related to cytosolic SYN and is therefore not detected in our western-blot analyses. This appears unlikely because of the high degrees of homology shown by SYN proteins of diverse species (Nour and Rabinowitz, 1992). Second, the cross-reacting 40- and 44-kD proteins may have some structural similarity to SYN but do not have this catalytic activity. In this regard, leaf mitochondria contain significant amounts of T-protein (subunit M_r of approximately 41,000) as part of the GDC complex (Bourguignon et al., 1993). T-protein also binds tetrahydrofolate and has some structural similarity to the SYN domains of mammalian C_1-tetrahydrofolate synthases (Kopriva et al., 1995). It should be noted, however, that the expression of T-protein in pea seedlings is strongly enhanced by light (Bourguignon et al., 1993), whereas the amount of the 44-kD, SYN-Ab-reacting protein in pea leaves decreased on exposure to light (Fig. 7). Third, the 40- and 44-kD proteins are structurally related to cytosolic SYN and represent unique mitochondrial forms of this enzyme. Because the SYN activities of yeast and mammalian mitochondria are integral parts of a trifunctional protein, the minimal structural requirements for a monofunctional SYN in plant mitochondria remain to be determined. It is clear that these aspects of mitochondrial folate metabolism require more detailed study.

The data in Figure 6 show that greening of etiolated pea seedlings was accompanied by increased levels of DHY and CYC activities in whole-leaf extracts. This treatment did not affect the specific activities of DHY or CYC in mitochondrial extracts (Table VI). These conditions resulted in a progressive loss of SYN activity in whole-leaf and -mitochondrial extracts (Fig. 6; Table VI). Western-blot analyses (Figs. 7 and 8) showed that greening had similar effects on the levels of the cross-reacting proteins. It follows that greening was accompanied by an enhancement in the synthesis or turnover of these folate-dependent enzymes. During the development of photorespiration in greening tissues, the expression of two other folate-dependent enzymes, GDC and SHMT, is strongly up-regulated (for review, see Cossins and Chen, 1997). As GDC catalyzes the mitochondrial formation of 5,10-CH_2-H_4PteGlu, it follows that greening will provide a substrate for mitochondrial DHY and SHMT. It is likely that most of this folate substrate will be utilized in Ser biosynthesis, since the specific activity of SHMT is about 15-fold higher than that of DHY in pea leaf mitochondria (Neuburger et al., 1996). Conceivably, the metabolic role of mitochondrial DHY and CYC is the conversion of 5,10-CH_2-H_4PteGlu to 10-HCO-H_4PteGlu, which is needed for synthesis of formylmethionyl-tRNA. It is noteworthy that 10-HCO-H_4PteGlu is a component of the mitochondrial folate pool (Table I). The relatively low levels of mitochondrial SYN activity (Table VI) suggest that the activation of formate is probably a minor route for 10-HCO-H_4PteGlu biosynthesis.

During photorespiration Ser is exported by mitochondria and converted to glycerate by peroxisomal enzymes (Douce and Neuburger, 1989). Since the cytosolic compartment of leaves contains SHMT (Neuburger et al., 1996), it follows that some of this exported Ser may be available for the biosynthesis of 5,10-CH_2-H_4PteGlu$_n$. In other eukaryotes, cytosolic pools of this folate have importance in the formation of thymidylate, purines, Met, and choline (Schirch, 1984). In mammalian cells the utilization of 5,10-CH_2-H_4PteGlu$_n$ in purine biosynthesis is facilitated by a functional interaction between cytosolic SHMT and C_1-tetrahydrofolate synthase (Strong and Schirch, 1989). There is still relatively little information on the biosynthesis of purines in plants (Cossins and Chen, 1997), but it is conceivable that green tissues generate the 10-HCO-H_4PteGlu needed in this pathway by cytosolic DHY-CYC activity. Thus, during photorespiration some of the Ser formed in the mitochondria may enter a cytosolic pathway that generates C-1-substituted folates for other pathways of one-carbon metabolism.

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