Effects of tetrahydrofolate polyglutamates on the kinetic parameters of serine hydroxymethyltransferase and glycine decarboxylase from pea leaf mitochondria

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Plant tissues contain highly conjugated forms of folate. Despite this, the ability of plant folate-dependent enzymes to utilize tetrahydrofolate polyglutamates has not been examined in detail. In leaf mitochondria, the glycine-cleavage system and serine hydroxymethyltransferase, present in large amounts in the matrix space and involved in the photorespiratory cycle, necessitate the presence of tetrahydrofolate as a cofactor. The aim of the present work was to determine whether glutamate chain length (one to six glutamate residues) influenced the affinity constant for tetrahydrofolate and the maximal velocities displayed by these two enzymes. The results show that the affinity constant decreased

by at least one order of magnitude when the tetrahydrofolate substrate contained three or more glutamate residues. In contrast, maximal velocities were not altered in the presence of these substrates. These results are consistent with analyses of mitochondrial folates which revealed a pool of polyglutamates dominated by tetra and pentaglutamates. The equilibrium constant of the serine hydroxymethyltransferase suggests that, during photorespiration, the reaction must be permanently pushed toward the formation of serine (the unfavourable direction) to allow the recycling of tetrahydrofolate necessary for the operation of the glycine decarboxylase T-protein.

INTRODUCTION

One-carbon metabolism in cells is mediated by a variety of folate derivatives (Cossins, 1984; McGuire and Coward, 1984). There is now strong evidence (McGuire and Coward, 1984; Cossins, 1987; Schirch and Strong, 1989) that the physiological forms of folate are γ -glutamyl-linked polyglutamates. Differences in glutamyl chain length, ranging from one to eight, have been reported for a variety of organisms, including plants (Kisliuk, 1981; McGuire and Coward, 1984; Zheng et al., 1992). Furthermore, there is some evidence that polyglutamate chain length may be influenced by conditions that alter the steady state of one-carbon metabolism (Kisliuk, 1981; Eto and Krumdieck, 1982). It is also clear that a number of folate-dependent enzymes display greater affinities for the polyglutamate forms of their substrates than for the corresponding monoglutamates (McGuire and Coward, 1984; Schirch and Strong, 1989). Among these enzymes, serine hydroxymethyltransferase (SHMT), which catalyses the interconversion of serine and glycine with tetrahydropteroyl-polyglutamate ($H_4PteGlu_n$) serving as the one-carbon carrier, has been extensively studied from a number of animal tissues (Schirch, 1984). Kinetic studies of liver SHMT show that affinity for the folate substrate increases when the polyglutamate chain contains more than three glutamic acid residues (Strong et al., 1990).

Apart from some early studies on the folate-dependent transmethylation of homocysteine (Cossins, 1980) the polyglutamate specificities of plant folate enzymes have not been examined. Two of these enzymes, SHMT and the T-protein of the glycine-cleavage complex, are present in large amounts in the matrix space of higher plant leaf mitochondria (Bourguignon et al., 1988). The glycine-cleavage complex catalyses the oxidation

of glycine in the presence of NAD^+ and $H_4PteGlu_n$ into CO_2 , $NADH$, NH_3 and $CH_2-H_4PteGlu_n$. During photorespiration, SHMT catalyses the reversible conversion of a second molecule of glycine and $CH_2-H_4PteGlu_n$ into serine and $H_4PteGlu_n$. Presumably these two reactions share a common pool of $H_4PteGlu_n$ and are therefore interdependent, owing to the recycling of this cofactor. Considering the high rate of glycine oxidation in leaf mitochondria (Oliver et al., 1990a) and the relatively low content of folate compounds in leaves (Imeson et al., 1990; Zheng et al., 1992) it is clear that the availability of $H_4PteGlu_n$ for glycine decarboxylase and its recycling through the SHMT reaction may be a critical step for glycine oxidation during photorespiration. Recent work (Imeson et al., 1990) has shown that the major $PteGlu_n$ derivatives of pea leaves are diglutamate (46%), pentaglutamate (38%) and tetraglutamate (15%). Considering the variability of these chain lengths and their possible role in determining the affinity of SHMT and glycine decarboxylase for $H_4PteGlu_n$, it follows that this chain may have a key role in controlling the velocity and the co-ordination of these two reactions.

In the present study we have measured the affinity of SHMT and the glycine decarboxylase complex for a series of $H_4PteGlu_n$ substrates ($n = 1-6$). In addition we have determined the polyglutamate chain distributions in the folate pool of isolated pea leaf mitochondria.

MATERIALS AND METHODS

Material

Pea (*Pisum sativum* L. var. Douce Provence) plants were grown from seeds in vermiculite for 15 days under a 12 h photoperiod at 22 °C. From approximately 1 kg of fully expanded leaves,

Abbreviations used: SHMT, serine hydroxymethyltransferase; $H_4PteGlu_n$, 5,6,7,8-tetrahydropteroylglutamate (tetrahydrofolate) with n glutamate residues; $CH_2H_4PteGlu_n$, 5,10-methylenetetrahydropteroylglutamate (methylenetetrahydrofolate) with n glutamate residues; $PteGlu_n$, pteroylglutamate (folate) with n glutamate residues; DTT, dithiothreitol.

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mitochondria were isolated and purified as previously described (Douce et al., 1987) using a self-generating gradient of Percoll.

Preparation of the matrix extract

Pea leaf mitochondria (about 100 mg of protein) were diluted in 100 ml of lysis buffer containing 5 mM Mops, 5 mM Tris, 1 mM β -mercaptoethanol, 1 mM EGTA, 20 μ M pyridoxal phosphate and 1 mM *n*-octyl β -D-glucopyranoside (Calbiochem), pH 7. Total release of the matrix proteins was achieved as previously described by three cycles of freezing and thawing (Neuburger et al., 1986). This procedure breaks about 98 % of the mitochondria. The suspension of broken mitochondria was centrifuged at 100 000 *g* for 2 h to remove all the mitochondrial membranes. The supernatant was then filtered on a Diaflo membrane XM-300 using an Amicon stirred cell.

Purification of the mitochondrial SHMT

The XM-300 matrix extract was applied to a column of Sephacryl S-300 (Superfine grade; Pharmacia) equilibrated in 50 mM KCl/5 mM Mops/5 mM Tris/2 mM β -mercaptoethanol/1 mM EGTA, pH 7.5 (buffer A). The column, connected with a Pharmacia f.p.l.c. system, was eluted with the same buffer at 4 °C (flow rate 0.5 ml/min; fraction size 2 ml). P-protein of the glycine-cleavage complex and the SHMT were eluted together (Bourguignon et al., 1988). Fractions containing the SHMT were concentrated and applied to a DEAE-cellulose column equilibrated with buffer A. Under these conditions the SHMT, in contrast with the P-protein, was not retained and was collected just after the bed volume of the column. Fractions containing SHMT were then applied to a CM-cellulose column previously equilibrated with 10 mM KH_2PO_4 /1 mM EGTA/1 mM dithiothreitol (DTT)/1 mM β -mercaptoethanol, pH 6.8. The protein was eluted with a linear KCl gradient (0–0.5 M) in the same medium, and SHMT was collected at about 0.2 M KCl. At this stage of purification the SHMT was almost totally pure, as shown by gel electrophoresis. The SHMT was dialysed and concentrated in the above buffer at 3 mg/ml.

Determination of the SHMT activity

The rate of the reaction was monitored by trapping labelled formaldehyde derived from labelled CH_2 - H_4PteGlu produced by SHMT activity (see the following equations) as a dimedon complex and extracting it into toluene (Taylor and Weissbach, 1965). The assay mixture contained 20 mM KH_2PO_4 , pH 7.4, 1 mM EGTA, 2 mM DTT, 1 mM β -mercaptoethanol, 4 mM L-[2,3- ^3H]serine (2.5×10^4 Bq/ μ mol) and enzyme. This mixture was maintained at 37 °C under argon for 2 min before the reaction was initiated by addition of various concentrations of $\text{H}_4\text{PteGlu}_n$ and kept under argon during all the periods of incubation.

At various times, samples of 0.2 ml were removed. The reaction was terminated by the addition of 150 μ l of 1 M sodium acetate, pH 4.5, followed by 100 μ l of 0.1 M formaldehyde and 150 μ l of 0.4 M dimedon. The sample was then heated for 5 min at 100 °C and the [^3H]formaldehyde–dimedon complex was extracted by vigorous shaking with 3 ml of toluene. After centrifugation for 10 min, 2 ml of the upper phase were removed for radioactivity counting in an Intertechnique SL 4000 liquid-scintillation counter. In preliminary experiments we verified that extraction of formaldehyde with dimedon was virtually complete, and a second addition of dimedon to the aqueous phase did not appreciably improve the yield of formaldehyde. For the determination of the

equilibrium constant of the reaction catalysed by SHMT, the procedure was the same, except that various serine/glycine ratios were present in the assay medium. The reaction was allowed to proceed for 60 and 80 min before it was terminated.

Determination of the glycine decarboxylase complex activity

Glycine oxidation was assayed at 25 °C by measuring the formation of NADH that was dependent upon the presence of both glycine and H_4PteGlu (Neuburger et al., 1986). The standard reaction mixture contained, in a total volume of 450 μ l: 20 mM KH_2PO_4 , pH 7.2, 1 mM EGTA, 1 mM β -mercaptoethanol, 10 μ M pyridoxal phosphate, 2 mM NAD, 20 mM glycine and 7 μ l of the matrix extract (16 mg/ml). The medium and all the solutions were maintained under a stream of argon to minimize the oxidation of H_4PteGlu . The reaction was started by the addition of H_4PteGlu . The rate of NADH formation was monitored at 340 nm using a Kontron (Uvikon 810) spectrophotometer.

Preparation of $\text{H}_4\text{PteGlu}_n$

PteGlu_n ($n = 2$ –6) were obtained from Dr. B. Schircks Laboratories, Jona, Switzerland, or synthesized ($n = 2$, $n = 5$) according to the method of Krumdieck and Baugh (1980). These compounds were reduced to $\text{H}_4\text{PteGlu}_n$ with KBH_4 according to the method of Scrimgeour and Vitols (1966); 10 mg of PteGlu_n were dissolved in 3 ml of 66 mM Tris, pH 7.8, and 15 mg of KBH_4 were added to this mixture. After 10 min the excess of KBH_4 was removed by addition of 0.3 ml of 1 M HCl and the solution was then neutralized with 1 M NaOH. After addition of 10 ml of a buffer A containing 0.13 M ammonium acetate, pH 6.9, plus 0.2 M β -mercaptoethanol, $\text{H}_4\text{PteGlu}_n$ was separated from other folates by chromatography on a Mono Q (Pharmacia) column, previously equilibrated with buffer A, and coupled to an f.p.l.c. system. $\text{H}_4\text{PteGlu}_n$ was eluted with a linear gradient of sodium acetate, pH 6.9 (0.13–2 M). $\text{H}_4\text{PteGlu}_n$ were identified by their absorption spectrum with a maximum at 299 nm. Fractions containing $\text{H}_4\text{PteGlu}_n$ were pooled and freeze-dried. $\text{H}_4\text{PteGlu}_n$ was adjusted to a final concentration of 5 mM in 10 mM Tris (pH 7)/0.1 M β -mercaptoethanol and kept in the dark under argon.

Determination of the glutamate chain length in the mitochondrial folate pool

Mitochondria were suspended in 2 ml of 10 mM KH_2PO_4 , pH 7, containing 50 mM β -mercaptoethanol and were heated for 10 min at 100 °C to inactivate folate hydrolase. The extract was centrifuged and the supernatant was adjusted to pH 1 with 5 M HCl. This extract was then subjected to a number of acid–base and Zn/HCl treatments to cleave the folylpolyglutamates to *p*-aminobenzoylpolyglutamates as described by Shane (1986). These derivatives were then converted into azo dyes of naphthylethylenediamine, purified on columns of Bio-Gel P2 and reconverted into *p*-aminobenzoylpolyglutamate by a second Zn/HCl treatment (Shane, 1986). The extracts were then concentrated under vacuum, dissolved in water and adjusted to pH 6.5 to remove $\text{Zn}(\text{OH})_2$. After centrifugation, the extracts were adjusted to 6 ml and Millipore-filtered before the various polyglutamate species were separated by h.p.l.c. H.p.l.c. was carried out on a Varian system as described by Imeson et al. (1990). The quantity of each *p*-aminobenzoylpolyglutamate species was calculated by reference to standard calibration curves. Authentic *p*-aminobenzoylpolyglutamate standards

(mono- to penta-glutamate) were obtained from Dr. B. Schircks Laboratories. Recovery of folates after cleavage, azo-dye formation and h.p.l.c. was estimated with PteGlu₅ and was $90 \pm 10\%$.

RESULTS

SHMT

The rate of the SHMT reaction was estimated as described by Taylor and Weissbach (1965) as the amount of formaldehyde (HCHO) derived from the CH₂-H₄PteGlu_n produced in the

Figure 1 Rates of HCHO production during the course of H₄PteGlu_n conversion into CH₂-H₄PteGlu_n by SHMT

The standard reaction mixture (1 ml) (see the Materials and method section) contained 75 μg of purified SHMT and was continuously maintained under an argon atmosphere. The reaction was initiated by addition of various concentrations of either H₄PteGlu₁, H₄PteGlu₂ or H₄PteGlu₄ as stated on the Figure. At each time point 0.2 ml of the reaction mixture was withdrawn and the amount of HCHO representative of the CH₂-H₄PteGlu_n concentration was determined as described in the Materials and methods section.

Figure 2 Effect of the initial concentration of (6S)-H₄PteGlu₁ and (6S)-H₄PteGlu₂ on the rate of the reaction catalysed by SHMT

Rates were calculated from data similar to those presented in Figure 1. The insert represents the double-reciprocal plots for (6S)-H₄PteGlu₁ and (6S)-H₄PteGlu₂.

Table 1 Affinity of SHMT for tetrahydrofolate and maximal velocities as a function of the glutamate chain length

The K_m values were calculated by assuming that only half of the tetrahydrofolate, i.e. the (6S) form of the coenzyme, was the substrate for the reaction

Number of glutamic acid residues	K_m (μM) for (6S)-H ₄ PteGlu _n	V_{max} (μmol · min ⁻¹ · mg of protein ⁻¹)
1	37.5	1.9
2	13.5	1.9
3	≤ 3.7	2
4	≤ 3.7	1.6
5	≤ 3.7	1.2
6	≤ 3.7	1.3

presence of serine and H₄PteGlu_n (see the Materials and methods section). As shown in Figure 1, the amount of formaldehyde formed by this reaction rapidly approached a stationary level, depending on the initial concentration of H₄PteGlu_n. Interestingly this final level was close to half the initial H₄PteGlu_n concentration present in the assay medium. Since the H₄PteGlu_n used in this study was obtained by a chemical reduction of folate with KBH₄, both stereoisomers (6R,6S) were present. The results in Figure 1 strongly suggest that only one stereoisomer, presumably the naturally occurring (6S) form, was substrate of the reaction. The reaction strongly decreased when this stereoisomer concentration was limiting. When the initial velocities were plotted against the concentrations of H₄PteGlu₁ and H₄PteGlu₂ present in the assay medium (Figure 2), we obtained hyperbolic saturation curves as predicted for Michaelis-Menten kinetics. It was not possible to get similar curves with (6S)-H₄PteGlu₃₋₆, because in these cases the enzyme appeared to be saturated with the lowest concentration of coenzyme (7.5 μM) used in the assays (see Figure 1c) and was always operating at its maximal velocity. Below this H₄PteGlu_n concentration the low signal/noise ratio prevented any reliable measurements. As shown in Table 1, the K_m values calculated for (6S)-H₄PteGlu_n ranged from 37 μM (Glu₁) to less than 3.7 μM (Glu₂₋₆), a variation of at least one order of magnitude. This Table also indicates that the maximal rate of reaction was not significantly changed by increasing the number of glutamate residues.

The recycling of H₄PteGlu_n, necessary to sustain glycine oxidation during photorespiration, implies that mitochondrial SHMT operates in the direction of serine formation. Therefore experiments were undertaken to determine the equilibrium constant of the reaction. Such measurements require long periods of incubation in order to reach equilibrium. However, when similar experiments as those described in Figure 1 were undertaken for longer periods of incubation in the presence of saturating amounts of H₄PteGlu_n, it was not possible to measure steady-state levels of HCHO (Figure 3). Indeed, under these conditions we obtained a biphasic curve (Figure 3). The rapid initial phase was independent of the H₄PteGlu_n concentration for values which saturated the enzyme activity, suggesting that the rapid accumulation of HCHO was catalysed by SHMT. In contrast, the second phase, corresponding to a slow accumulation of HCHO, was linear with time and its rate was dependent on the initial H₄PteGlu_n concentration. When times of incubation were increased to nearly 2 h, the amount of HCHO produced by the reaction far exceeded the initial H₄PteGlu_n concentration (result not shown). This result demonstrates a continuous recycling of H₄PteGlu_n. In fact it is well known that CH₂-H₄PteGlu_n is an

Figure 3 Rates of HCHO production during the course of H₄PteGlu_n conversion into CH₂-H₄PteGlu_n by SHMT

The standard reaction mixture (2.5 ml) contained 60 μg of SHMT and was maintained under an argon atmosphere during all the periods of incubation. The reaction was initiated by addition of H₄PteGlu₁ at various concentrations as indicated on the Figure. At each time point 0.2 ml of the reaction mixture was withdrawn and the amount of HCHO produced was determined as described in the Materials and methods section.

Table 2 Equilibrium constant of the reaction catalysed by SHMT

The amount of HCHO formed was measured after 60 and 80 min of incubation. The calculations were made by assuming that only half of the initial tetrahydrofolate concentration was substrate of the reaction. $K_{eq} = [\text{glycine}][\text{CH}_2\text{-H}_4\text{PteGlu}_n]/[\text{serine}][\text{H}_4\text{PteGlu}_n]$.

Initial concn.			HCHO formed		
(nmol · ml ⁻¹)	(μmol · ml ⁻¹)		(nmol · ml ⁻¹)		<i>K</i> _{eq}
(6 <i>S</i>)-H ₄ PteGlu _{<i>n</i>}	Glycine	Serine	60 min	80 min	
Glu ₁ 100	30	2	55	56.5	18.5
Glu ₁ 100	10	1	58	58	14
Glu ₁ 100	40	1	23	23	12
Glu ₁ 100	40	2	42	41	15
Glu ₄ 65	20	2	40	40	16

unstable compound which undergoes a spontaneous cleavage into H₄PteGlu_n and HCHO (Osborn et al., 1960). Therefore, according to the following equations, while HCHO accumulated in the medium (2), H₄PteGlu_n, resulting from the slow non-enzymic cleavage of CH₂-H₄PteGlu_n, was recycled by SHMT (1) at a rate which was dependent on the CH₂-H₄PteGlu_n concentration.

However, the linear increase of HCHO observed in Figure 3 was almost entirely suppressed when glycine at concentrations higher than 10 mM was added to the incubation medium. In this situation, steady-state levels of HCHO have been recorded after 60 and 80 min of incubation (Table 2). Indeed, in the presence of glycine, the back reaction catalysed by SHMT (1) recycled CH₂-H₄PteGlu_n at high rates to give serine and H₄PteGlu_n, a situation

Figure 4 Effect of the glutamate chain length on the affinity constant of the glycine decarboxylase complex for H₄PteGlu_n

Production of the matrix extract and the determination of the rates of glycine oxidation were as described in the Materials and methods section. K_m and V_{max} for each H₄PteGlu_n were calculated from double-reciprocal plots. Values are the averages of three or four determinations.

which competes favourably with the relatively slow non-enzymic cleavage of CH₂-H₄PteGlu_n (2). Within these experimental conditions it was possible to calculate the equilibrium constant of the mitochondrial SHMT (Table 2). After 60 min of incubation under argon the reaction was at equilibrium, and longer periods of incubation did not change the final concentration of the substrates. Taking account of the fact that only the (6S) stereoisomer participates in the reaction the calculated values ($K_{eq} = [\text{glycine}][\text{CH}_2\text{-H}_4\text{PteGlu}_n]/[\text{serine}][\text{H}_4\text{PteGlu}_n]$), varied between 12 and 19, a range of values in agreement with those previously reported for mammalian SHMT (Schirch et al., 1977). As also shown in Table 2, the equilibrium constant was the same for H₄PteGlu₁ and H₄PteGlu₄, indicating that increasing the number of glutamate residues affects in a similar manner both forward and backward reactions of SHMT, as expected for two molecules acting as identical substrates.

Glycine decarboxylase

The rate of glycine oxidation was determined by monitoring the formation of NADH in the presence of glycine, NAD⁺, H₄PteGlu_n and a matrix extract (Neuburger et al., 1986). Since the activity of this complex relied on the cooperation of four distinct proteins, the P-, H-, T- and L-proteins (Oliver et al., 1990b), we verified, in separate control experiments, that these subunits were present in the optimal stoichiometry. Indeed, a further addition of either P-, H-, T- or L-proteins to the matrix extract did not change the rate of NADH production, indicating that none of these enzymes was limiting. Therefore, as previously demonstrated by Bourguignon et al. (1988), the initial increase of NADH concentration upon addition of limiting amounts of H₄PteGlu_n permits measurements of the T-protein activity. The H₄PteGlu_n saturation curves were hyperbolic for all of the polyglutamate forms of H₄PteGlu_n (results not shown). The K_m values calculated for each of these forms are shown in Figure 4. The values arranged from 17 μM (monoglutamate) to 0.5 μM (tetra- and penta-glutamate) assuming that only the (6S) stereoisomer was substrate of the reaction. The maximal velocities calculated from Lineweaver-Burk plots were not significantly affected by the number of glutamate residues present in the folate substrate (Figure 4).

Since our results indicate that H₄PteGlu_n with a polyglutamate chain of more than two residues presented a much higher affinity

Figure 5 Distribution of polyglutamate derivatives in the folate pool of pea leaf mitochondria

Mitochondrial folates were cleaved and converted into *p*-aminobenzoylpolyglutamates as described in the Materials and methods section. These derivatives were separated by h.p.l.c. on a Whatman Partisil SAX-10. Extracts □ and ▨ were obtained from the same mitochondrial preparation, and the total folate recovered were respectively 0.97 nmol/mg of protein and 1.28 nmol/mg of protein. Extract ▩ was from a second mitochondrial preparation, and the total folate recovered was 0.39 nmol/mg of protein.

for the glycine decarboxylase and the SHMT than monoglutamate species, it was of interest to determine the glutamate chain length of pea leaf mitochondria folate compounds.

Determination of the glutamate chain length in pea leaf mitochondria extracts

Three determinations of the glutamate chain length were undertaken with two different matrix extracts from purified pea leaf mitochondria. The *p*-aminobenzoylpolyglutamates derived by chemical cleavage of the PteGlu_n compounds were isolated by h.p.l.c. using the method of Shane (1986) (see the Materials and methods section). As shown in Figure 5, the major polyglutamates were penta- and tetra-glutamates which accounted for 55 and 25% respectively of the *p*-aminobenzoylpolyglutamates recovered in these analyses. We did not find any evidence for longer chain length (Glu₆, Glu₇) in the pea leaf mitochondrial extracts analysed by h.p.l.c. These mitochondrial data are in agreement with earlier analyses of pea leaf extracts, where tetra- and penta-glutamates were detected as the principal derivatives (Imeson et al., 1990). Although the distribution of each polyglutamate species was comparable in the three different pea mitochondrial extracts, the total folate content, when expressed on a per-mg-of-protein basis, was found to be lower in one of the extracts (0.39 nmol/mg) than in the other two (0.97 and 1.28 nmol/mg) (see the legend of Figure 5). It is possible that some folate was lost during the extraction and cleavage procedure, although the yield of recovery with this method was approx. 90% (Imeson et al., 1990; Eto and Krumdieck, 1981). It is also possible that mitochondria from different batches of leaves may have variable levels of total folate, as noted for leaf extracts by Zheng et al. (1992) or different levels of folate-dependent enzymes containing bound folate.

DISCUSSION

Our results indicate that SHMT and the T-protein of the glycine cleavage complex display higher affinities for H₄PteGlu_n sub-

strates containing more than three glutamic residues. However, the T-protein did not show appreciably lower *K_m* values when the tetra- and penta-glutamate substrates were provided (see Figure 4). As these latter compounds are the major PteGlu_n of pea mitochondria (see Figure 5), it seems likely that other folate-dependent enzymes within this organelle, including SHMT, may have a preference for these more highly conjugated PteGlu_n. This possibility is supported by a number of kinetic studies of mammalian folate-dependent enzymes (McGuire and Coward, 1984; Schirch and Strong, 1989), where affinity for the folate substrate tends to reflect the chain length of the native folate pool. The importance of polyglutamate derivatives in leaf mitochondria folate metabolism is also consistent with earlier observations indicating that mutations affecting the generation of conjugated folates resulted in auxotrophies for methionine and products of one-carbon metabolism such as thymidine and purine (Cossins, 1987; Moran et al., 1976).

The conjugate nature of pea mitochondrial folates may also have a role in the retention of these derivatives within the matrix space. In mammalian cells monoglutamyl folates are readily transported across cellular membranes. On the other hand, permeability to polyglutamyl folates is considerably less, and these derivatives are usually retained by living cells (McGuire and Bertino, 1981). It is also clear from studies of cultured mammalian cells that mitochondrial folate accumulation is dependent on their ability to generate polyglutamates *in situ*. If this holds true for plant mitochondria, it follows that folylpolyglutamate synthetase must also be present in the matrix space.

Another function of the polyglutamate chain of plant mitochondrial folates may be the co-ordination of related enzyme activities such as those catalysed by the T- and SHMT proteins. Folylpolyglutamates are known to increase the efficiency of sequential folate-dependent enzymes by enhancing the 'channeling' of intermediates between the active sites of protein complexes (McGuire and Bertino, 1981; MacKenzie, 1984; Schirch and Strong, 1989). In other words, folate polyglutamate intermediates do not equilibrate with the bulk solvent but are directly transferred between the active sites of the different proteins, a situation which facilitates the establishment of the steady state. In this regard, CH₂-H₄PteGlu_n produced by the glycine decarboxylase T-protein and required in the SHMT reaction may not undergo spontaneous cleavage in free solution if both of these proteins are closely associated to allow 'channeling' of this compound.

It is also conceivable that changes in the glutamyl chain length of the PteGlu_n derivatives could play a key role in the binding of these derivatives to folate-dependent enzymes. Under conditions of high glycine decarboxylase activity, an increase in the glutamyl conjugation of H₄PteGlu_n would favour a better access to these enzymes. Indeed there is some evidence, based on the amino acid sequences of mammalian and yeast C-1 tetrahydrofolate synthase (Whitehead and Rabinowitz, 1988; Nour and Rabinowitz, 1992), that the negatively charged α-COO⁻ groups of the poly-γ-glutamate chain could bind at specific points, such as basic groups of the proteins. In this connection, arginine residues are necessary for the binding of H₄PteGlu_n to sheep liver SHMT (Usha et al., 1992). Quantification of the T- and SHMT proteins indicates that they represent respectively 10 and 2% of the soluble mitochondrial proteins (respectively 4 and 1% of the total proteins) (Bourguignon et al., 1988; Oliver et al., 1990b). On the basis of the molecular masses of these proteins and assuming that the tetrameric SHMT binds one folate molecule per subunit and the monomeric T proteins binds one folate molecule, it can be estimated that both proteins are potentially able to bind twice the folate content of the matrix space (see Figure 5). Thus we

conclude that the polyglutamates of leaf mitochondria are probably largely bound to the active sites of these two major folate-dependent enzymes. Such a situation could also allow a better protection of these very labile and oxidizable tetrahydrofolate compounds. We also visualize minor pools of monoglutamyl folate derivatives that may arise in the dihydrofolate synthetase reaction and, after reduction, are utilized by folylpolyglutamate synthetase. The former activity is present at very low levels in pea seedling mitochondria (Iwai and Ikeda, 1975). By analogy with other species (Shane et al., 1989) it might be assumed that plant mitochondria also contain some folylpolyglutamate synthetase activity. These points are currently under investigation.

Our results indicate that recycling of $\text{CH}_2\text{-H}_4\text{PteGlu}_n$ into $\text{H}_4\text{PteGlu}_n$, and consequently the operation of the overall glycine-cleavage system, requires that the SHMT reaction is continuously driven towards the production of serine. Since the equilibrium constant of the reaction does not favour this direction, we must speculate that the $\text{CH}_2\text{-H}_4\text{PteGlu}_n/\text{H}_4\text{PteGlu}_n$ ratio in the matrix space is high and/or the serine produced is rapidly exported out of the mitochondria. Steady-state $\text{CH}_2\text{-H}_4\text{PteGlu}_n/\text{H}_4\text{PteGlu}_n$ ratios have been measured during the time course of glycine oxidation by matrix extracts of pea leaf mitochondria (Bourguignon et al., 1988) and the values ranged from 0.4 to 0.7, depending of the initial $\text{H}_4\text{PteGlu}_n$ concentration. Therefore it seems that the mitochondrial concentration of serine is a key regulatory point of the glycine-cleavage system and that the rate of serine export is a critical step for glycine oxidation. At present there are only limited data concerning the transport of serine through the inner membrane of plant mitochondria (Oliver, 1987), but there is good evidence from a mutant of barley that accumulates serine that there is also concurrent build-up (and presumably inhibition of the oxidation) of glycine (Murray et al., 1989). Finally, it is also possible that SHMT, glycine decarboxylase and pteroylpolyglutamate form a supramolecular complex and do not behave as separate components in the matrix space. One consequence of this organization would be the 'channelling' of glycine. This point is currently under investigation.

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