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Interaction between glycine decarboxylase, serine hydroxymethyltransferase and tetrahydrofolate polyglutamates in pea leaf mitochondria

Fabrice REBEILLE,* Michel NEUBURGER and Roland DOUCE

CEA, Physiologie Cellulaire Végétale, CNRS No. 576, DBMS, CEN-G, F-38054 Grenoble Cédex 9, France

The aim of the present work was to further determine how the T-protein of the glycine-cleavage system and serine hydroxymethyltransferase (SHMT), two folate-dependent enzymes from pea leaf mitochondria, interact through a common pool of tetrahydrofolate polyglutamates ($H_4PteGlu_n$). It was observed that the binding affinity of tetrahydrofolate polyglutamates for these proteins continuously increased with increasing number of glutamates up to six residues. It was also established that, once bound to the proteins, tetrahydrofolate, a very O_2-sensitive molecule, was protected from oxidative degradation. The dissociation constants (K_d) of $H_4PteGlu_5$, the most predominant form of polyglutamate in the mitochondria, were approximately 0.5 μM for both T-protein and SHMT, whereas the K_d values of CH_2-$H_4PteGlu_5$ were higher, 2.7 and 7 μM respectively. In a matrix extract from pea leaf mitochondria, the maximal activity of the glycine-cleavage system was about 2.5 times higher than the maximal activity of SHMT. This resulted in a permanent disequilibrium of the SHMT-catalysed reaction which was therefore driven toward the production of serine and $H_4PteGlu_n$, the thermodynamically unfavourable direction. Indeed, measurements of the steady-state ratio of CH_2-$H_4PteGlu_n$/$H_4PteGlu_n$ ($n = 1$ or $n = 5$) during the course of glycine oxidation demonstrated that the methylene form accounted for 65–80% of the folate pool. This indicates that, in our *in vitro* experiments, CH_2-$H_4PteGlu_n$ with long polyglutamate chains accumulated in the bulk medium. This observation suggests that, in these *in vitro* experiments at least, there was no channelling of CH_2-$H_4PteGlu_5$ between the T-protein and SHMT.

INTRODUCTION

During photorespiration, mitochondria from plant leaves are capable of oxidizing glycine at high rates (Douce, 1985; Oliver et al., 1990a). Glycine oxidation is catalysed by the glycine-cleavage system (Neuburger et al., 1986), according to eqn. (1). This system is extensively present in the matrix space of the mitochondria and consists of four different component proteins, referred to as P, H, T and L, acting as a multienzyme complex (Oliver et al., 1990b). The continuous operation of this reaction is ensured by NADH reoxidation through the mitochondrial electron-transport chain and by recycling of CH_2-$H_4PteGlu_n$ to $H_4PteGlu_n$ via a reversible reaction catalysed by serine hydroxymethyltransferase (SHMT) [eqn. (2)].

$$\text{Glycine} + NAD^+ + H_4PteGlu_n \rightarrow CH_2\text{-}H_4PteGlu_n + NADH + NH_3 + CO_2 \quad (1)$$

$$\text{Serine} + H_4PteGlu_n \leftrightarrow CH_2\text{-}H_4PteGlu_n + \text{glycine} \quad (2)$$

Glycine oxidation relies on two folate-dependent proteins, SHMT and the T-protein of the glycine-cleavage complex, both present in large amounts in the matrix space of higher-plant leaf mitochondria (Bourguignon et al., 1988). Considering the high rate of glycine oxidation (Oliver et al., 1990a), it is clear that the availability of $H_4PteGlu_n$ for glycine decarboxylase and its recycling through SHMT may be a critical step in this reaction.

There is now strong evidence that the physiological forms of folate are γ-glutamyl-linked polyglutamates (McGuire and Coward, 1984; Schirch and Strong, 1989; Imeson et al., 1990). It is also clear from studies on micro-organisms and animal tissues that a number of folate-dependent enzymes display greater affinity for the polyglutamate forms of their substrates than for the corresponding monoglutamates (McGuire and Coward, 1984; Schirch, 1984; Strong et al., 1990). In this connection, it has been proposed that the polyglutamate forms of folate increase the efficiency of sequential folate-dependent proteins by enhancing the 'channelling' of intermediates between the active sites (McGuire and Bertino, 1981; MacKenzie, 1984; Schirch and Strong, 1989).

In plants, little is known about the polyglutamate specificities of folate enzymes. In a previous paper (Besson et al., 1993) we have shown that both T-protein and SHMT from pea leaf mitochondria have a high affinity for $H_4PteGlu_n$ containing three or more glutamate residues. Analysis of mitochondrial folates revealed a pool of polyglutamate dominated by tetra- (25%) and penta- (55%) glutamates, and it was postulated that these long glutamate chains might play a key role in the binding of these derivatives to the enzymes (Besson et al., 1993). Furthermore, it was concluded from the determination of the SHMT equilibrium constant that the reversible reaction (2) must be permanently pushed out of equilibrium, toward the production of serine, to allow efficient recycling of $H_4PteGlu_n$. In the present article we further demonstrate how the T-protein and SHMT from pea leaf mitochondria interact through a common pool of $H_4PteGlu_n$ to maintain high rates of glycine oxidation.

Abbreviations used: SHMT, serine hydroxymethyltransferase; $H_4PteGlu_n$, 5,6,7,8-tetrahydropteroylglutamate (tetrahydrofolate) with n glutamate residues; CH_2-$H_4PteGlu_n$, 5,10-methylenetetrahydropteroylglutamate (methylenetetrahydrofolate) with n glutamate residues.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

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). Mitochondria were isolated and purified as previously described (Douce et al., 1987) using a self-generating gradient of Percoll. Preparation of matrix extracts and purification of mitochondrial SHMT and mitochondrial T-protein were undertaken as previously described (Neuburger et al., 1986; Bourguignon et al., 1988).

Determination of glycine decarboxylase and SHMT activities

Glycine oxidation was assayed at 30 °C by measuring the initial rate of NADH formation dependent on the presence of both glycine and $H_4PteGlu_n$. As previously described (Neuburger et al., 1986), the rate of NADH production was clearly biphasic. The initial velocity reflected the activity of the glycine-cleavage system whereas the lower steady-state rate reflected the rate of $H_4PteGlu_n$ recycling via SHMT activity (Bourguignon et al., 1988).

The rate of the reaction catalysed by SHMT was monitored at 30 °C as described by Besson et al. (1993).

Dissociation constants of $H_4PteGlu_5$ and CH_2-$H_4PteGlu_5$ for the T- and SHMT proteins

The T-protein is a basic (Bourguignon et al., 1993) unstable protein which is precipitated in an irreversible manner at pH 7.5 when its concentration exceeds 2 mg/ml. A careful examination of the amino acid sequence of the mature T-protein indicates that, besides aligned basic residues, this sequence contains an unusual amount of hydrophobic amino acids leading to strong interactions between the proteins (Bourguignon et al., 1993). However, it was observed that this protein could be stabilized by the addition of equimolar amount of H-protein, another component of the glycine-cleavage system, which suggests a strong interaction between these two enzymes. This point is currently under investigation. Under these conditions, T-protein concentrations up to 20 mg/ml could be readily obtained. Therefore H-protein was systematically added to the T-protein and we verified in separate experiments that $H_4PteGlu_5$ did not bind to the H-protein. SHMT (16 μg; 0.08 nmol) or T-protein (16 μg; 0.4 nmol) plus H-protein (6 μg; 0.4 nmol) was incubated in 500 μl of buffer A containing various concentrations of $^3H_4PteGlu_5$. The mixture was placed in a Millipore membrane filter system (Millipore Ultrafree-PF) with a cut-off at 10 kDa, as described above, and kept under a stream of argon. After 10 min the mixture was forced through the membrane at an argon pressure of 414 kPa, and the proteins retained on the filter were dissolved in 2 × 500 μl of buffer A. The radioactivity associated with the proteins was measured in an Intertechnique SL 4000 liquid-scintillation counter. In separate control experiments we verified that $^3H_4PteGlu_5$ did not bind to H-protein. The volume of buffer retained on the membrane was determined for each concentration of $^3H_4PteGlu_5$ in the absence of the T- and SHMT proteins, and the radioactivity associated with these volumes was subtracted from the data. CH_2-$^3H_4PteGlu_5$ was obtained by incubating $^3H_4PteGlu_5$ with 1 mM formaldehyde for 5 min before the addition of the proteins. We have verified in separate experiments that this concentration of formaldehyde did not affect the rate of glycine oxidation, and was sufficient, as shown by the change in A_{290}, to convert up to 100 μM $H_4PteGlu_5$ into CH_2-$H_4PteGlu_5$ completely in 5 min (Kallen and Jencks, 1966).

The dissociation constants

$$K_d = [E][S]/[ES] \quad (3)$$

and the number of binding sites were calculated by Scatchard plots and by fitting the experimental data to the theoretical hyperbolic equation resulting from the development of eqn. (3)

$$[ES] = ([S_o]+[n]+K_d)/2 - \sqrt{\{([S_o]+[n]+K_d)^2 - 4[n][S_o]\}}/2 \quad (4)$$

where [ES] is the measured amount of bound tetrahydrofolate, $[S_o]$ is the initial $H_4PteGlu_5$ (pro*S*) concentration, i.e. $[S_o]$ is half of the total $H_4PteGlu_5$ (pro*R*, pro*S*) initial concentration and [n] is the concentration of binding sites.

Determinations of CH_2-$H_4PteGlu_n$ produced during glycine oxidation

Proteins from a matrix extract (0.6 mg/ml) were placed in buffer A [20 mM KH_2PO_4 (pH 7.4), 1 mM EGTA, 1 mM 2-mercaptoethanol, 10 μM pyridoxal phosphate] and incubated in the presence of 10 mM [2-^{14}C]glycine (8.5 MBq/mmol), 2 mM NAD^+ and various concentrations of either $H_4PteGlu_5$ or $H_4PteGlu_1$. After 4 min, the reaction was stopped and the level of $H_4PteGlu_n$ was determined as described for the determination of SHMT activity.

Binding experiments with the matrix extract

Binding measurements were carried out by a 'forced dialysis' technique using a Millipore membrane system (Millipore, Ultrafree-PF) with a cut-off at 10 kDa. Matrix extract (7 μl; 20 mg/ml) was incubated under a stream of argon in 500 μl of buffer A. The mixture was placed in the Millipore membrane system, and 50 μM concentrations of various $H_4PteGlu_n$ species were added. After 10 min, the mixture was forced through the membrane at an argon pressure of 414 kPa. In order to remove the free $H_4PteGlu_n$ completely, the proteins retained on the membrane were resuspended in 500 μl of the same buffer and filtered again. After this final filtration step, proteins retained on the membrane were resuspended in 500 μl of buffer A and transferred to a cuvette to determine glycine decarboxylase activity. The rate of NADH production was measured after the addition of 2 mM NAD^+ and 20 mM glycine. The rates were compared with the maximal velocities obtained after addition of saturating amounts of $H_4PteGlu_n$.

Measurement of the rates of $H_4PteGlu_5$ oxidation

Portions (500 μl) of Buffer A containing 5 μM $H_4PteGlu_5$ were placed in test-tubes, well aerated by vigorous shaking and left to stand for various lengths of time. The 500 μl samples were then transferred to cuvettes to determine the ability of $H_4PteGlu_5$ to sustain glycine oxidation in the presence of 7 μl of matrix extract (20 mg/ml), 2 mM NAD^+ and 20 mM glycine. The rates were compared with the maximal velocities obtained under anaerobic conditions and with identical $H_4PteGlu_5$ and protein concentrations.

Alternatively, 5 μM $H_4PteGlu_5$ was incubated for 10 min under argon with 7 μl of matrix proteins (20 mg/ml). The proteins, with bound $H_4PteGlu_5$, were collected after two consecutive filtrations on a Millipore membrane system (10 kDa cut-off), as described above, in order to remove free $H_4PteGlu_5$. They were then transferred to 500 μl of buffer A in a test-tube, and the mixture was well aerated by vigorous shaking, and left to stand for various lengths of time. Each sample was then transferred to a cuvette to determine the rate of glycine oxidation in the presence of 2 mM NAD^+ and 20 mM glycine.

Preparation of $H_4PteGlu_n$

$PteGlu_n$ ($n = 2$–6) species were obtained from Dr. B. Schircks Laboratories, Jona, Switzerland. These compounds were reduced to 5,6,7,8-$H_4PteGlu_n$ with $NaBH_4$ by the method of Scrimgeour and Vitols (1966). $PteGlu_n$ (10 mg) was dissolved in 3 ml of 66 mM Tris/HCl, pH 8, and 15 mg of $NaBH_4$ was added. After 10 min, excess $NaBH_4$ was removed by addition of 0.3 ml of 1 M HCl and the solution was then neutralized with 1 M NaOH. After the addition of 10 ml of 0.13 M ammonium acetate buffer (pH 6.9) containing 0.2 M 2-mercaptoethanol, $H_4PteGlu_n$ was separated from other folates by chromatography on a Mono Q column (Pharmacia), previously equilibrated with the same buffer, and coupled to an f.p.l.c. system. All these operations were carried out under argon. $H_4PteGlu_n$ was eluted with a linear gradient of ammonium acetate (pH 6.9) (0.13–2 M). $H_4PteGlu_n$ species were identified by their absorption spectra with a maximum at 299 nm. Fractions containing $H_4PteGlu_n$ were pooled and lyophilized. $H_4PteGlu_n$ was adjusted to a final concentration of 5 mM in 10 mM Tris/HCl (pH 7.4) and 0.1 M 2-mercaptoethanol, and kept in darkness under argon.

For the preparation of $^3H_4PteGlu_5$, 10 mg of $PteGlu_5$ was reduced with 2.5 mg of NaB^3H_4 (37 MBq/mmol) and processed as described above. At alkaline pH, the protons located at the N-5 and N-8 positions readily exchange with the protons from water. Therefore, after chromatography, the fractions containing $^3H_4PteGlu_5$ were adjusted to pH 8 before lyophilization and we assumed in the calculations that there was no labelling at positions N-5 and N-8. This assumption was supported by the stability of the specific radioactivity of these derivatives which was constant for over a week.

RESULTS

Pea leaf mitochondria contain two major folate-dependent proteins: the T-protein of the glycine decarboxylase complex and SHMT (Bourguignon et al., 1988). The role of the $H_4PteGlu_n$ polyglutamate chain in the binding of this cofactor to folate-dependent enzymes was estimated with soluble proteins extracted from pea leaf mitochondria. In these experiments soluble proteins from the matrix space were first incubated for 10 min with various $H_4PteGlu_n$ species (Glu = 1 to 6), then filtered on a membrane which retains proteins of molecular mass higher than 10 kDa (see the Materials and methods section). The binding of $H_4PteGlu_n$ to the proteins was indirectly estimated by determining the ability of the protein mixture to catalyse glycine oxidation in the presence of glycine and NAD^+. The measured rates were then compared with the maximal velocities obtained with saturating amounts of $H_4PteGlu_n$ (control experiments). We have verified that the $V_{max.}$ was not affected by the length of the polyglutamate chain (Besson et al., 1993). As shown in Figure 1(a), $H_4PteGlu_1$ did not bind significantly to the matrix proteins during the filtration experiments, but increases in polyglutamate chain length resulted in almost proportional increases in binding. In these experiments the matrix proteins were washed once before glycine decarboxylase activity was determined (see the Materials and methods section). However, the amount of $H_4PteGlu_n$ attached to the proteins decreased when the number of washes was increased (results not shown), suggesting that the binding of these polyglutamate species was not very tight. In this regard, it should be pointed out that $H_4PteGlu_n$ present in the matrix space of the mitochondria was lost during the protein-extraction procedure, as the addition of $H_4PteGlu_n$ was an absolute requirement for the measurement of glycine decarboxylase activity. As already reported for mammalian SHMT (Strong et al., 1989), binding of $H_4PteGlu_n$ increased with the number of glutamates, which supports the idea that it was correlated with the negative charges on the glutamate chain.

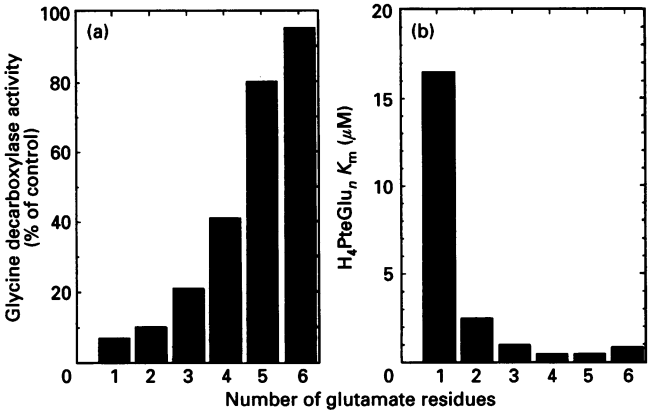

Figure 1 Effect of the number of glutamate residues on (a) $H_4PteGlu_n$ binding to matrix proteins from pea leaf mitochondria and (b) catalytic efficiency of $H_4PteGlu_n$ for glycine oxidation

(**a**) The matrix extract was incubated under a stream of argon at room temperature in the presence of 50 μM $H_4PteGlu_n$ for 10 min, filtered through a 10 kDa cut-off Millipore membrane, resuspended in the initial buffer and filtered again. $H_4PteGlu_n$ binding was indirectly estimated by measuring the ability of the matrix proteins to sustain glycine oxidation in the presence of NAD^+ and glycine. The results are expressed as percentage of the maximal activity recorded in the presence of a saturating amount of $H_4PteGlu_n$. (**b**) K_m for each $H_4PteGlu_n$ was determined from double-reciprocal plots and are the means of three different determinations.

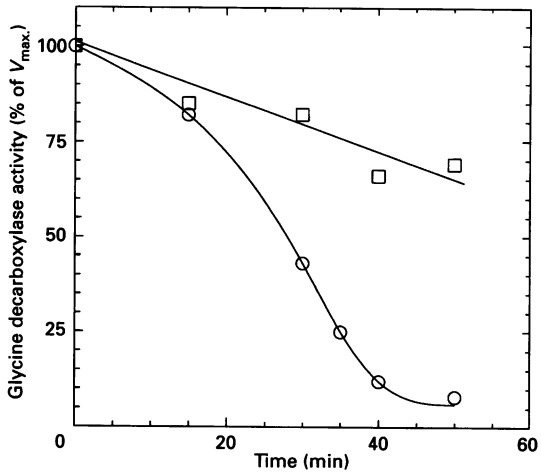

Figure 2 Oxidative degradation of $H_4PteGlu_5$ when either free in solution or bound to the matrix proteins

$H_4PteGlu_5$ (5 μM) (○) or $H_4PteGlu_5$ (5 μM) previously bound to 0.14 mg of protein (see the Materials and methods section) (□) was placed in 500 μl of a well-aerated buffer. After various times, $H_4PteGlu_5$ degradation was estimated by measuring the ability of this cofactor to sustain glycine oxidation. The results are expressed as percentage of the maximal velocity of the reaction measured at $t = 0$ under anaerobic conditions.

Surprisingly, there was no strict correlation between $H_4PteGlu_n$ binding and the catalytic efficiency (K_m) of these species for the glycine decarboxylase complex plus SHMT. Indeed, as previously reported (Besson et al., 1993) and shown in Figure 1(b), near-maximal catalytic efficiency was obtained for $H_4PteGlu_3$ [although binding of this molecular species was rather weak (Fig. 1a)], and did not significantly increase with increasing number of glutamate residues.

$H_4PteGlu_n$ is a very labile molecule which undergoes rapid

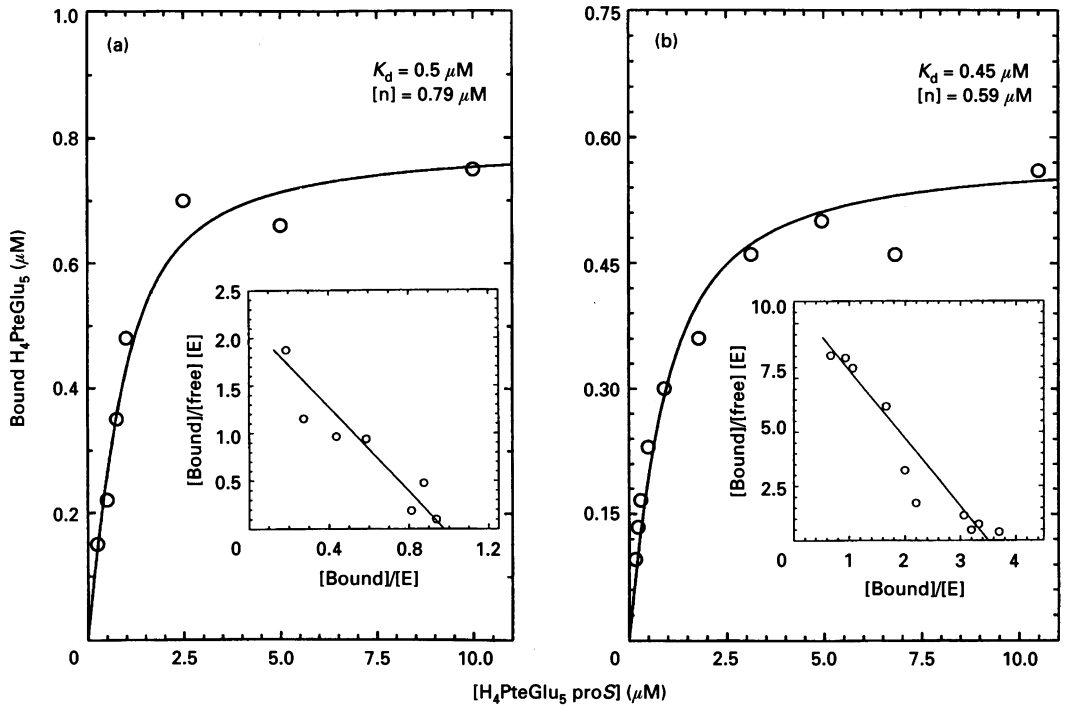

Figure 3 Dissociation constants of $H_4PteGlu_5$ for the T-protein (a) and SHMT (b) from pea leaf mitochondria

The concentrations of T-protein and SHMT were respectively 0.8 (monomer) and 0.16 (tetramer) μM. The curves were determined by computer-fitting the experimental data using the theoretical hyperbolic curve defined in the Materials and methods section. The insets show Scatchard plots calculated from the experimental values. [n], concentration of binding sites.

degradation when in contact with O_2. Thus it must be protected from oxidation in the matrix space of mitochondria, and binding of $H_4PteGlu_n$ to folate-dependent proteins may play a protective role against the degradative effects of O_2. To test this hypothesis, we measured the rate of degradation of $H_4PteGlu_n$ when either free in solution or bound to matrix proteins. As it was previously shown that the mitochondrial folate pool is dominated by the pentaglutamate forms (Besson et al., 1993), the experiments were carried out with $H_4PteGlu_5$. As shown in Figure 2, $H_4PteGlu_5$, when added to a well-oxygenated buffer, lost over time its ability to sustain the maximum rate of glycine oxidation. Indeed, with an initial $H_4PteGlu_5$ concentration of 5 μM, the rate of glycine oxidation was only 50 % of the original rate after 25 min. As the apparent K_m of $H_4PteGlu_5$ for this reaction was about 0.5–1 μM, our results indicate that 80–90 % of the molecule was degraded within this time. In contrast, when $H_4PteGlu_5$ was attached to matrix proteins before being added to the oxygenated buffer, the rate of glycine oxidation decreased by only 15–20 % during the same period of time and under the same experimental conditions (Figure 2). These results indicate that $H_4PteGlu_5$ bound to folate-dependent proteins was protected in some way from oxidation, which increased its lifetime by a factor of 4–5. This is a minimum value as a slow release of $H_4PteGlu_5$ from folate-dependent proteins might also explain the observed decrease in the rate of glycine oxidation. Indeed, once released from the proteins, folate underwent rapid oxidation.

Figures 1(a) and 1(b) indicate that tetrahydrofolate with long polyglutamate chains bound more tightly to the glycine decarboxylase complex plus SHMT than species with short polyglutamate chains. However, the reactions catalysed by these enzymes rely on two different folate species, i.e. $H_4PteGlu_n$ and CH_2-$H_4PteGlu_n$, which might have different binding affinities. Therefore the dissociation constants of [6,7-^{3}H]$H_4PteGlu_5$ and [6,7-^{3}H]CH_2-$H_4PteGlu_5$ with purified T-protein and SHMT were determined. As shown in Figure 3 and Table 1, the K_d values of $H_4PteGlu_5$ for T-protein and SHMT were almost identical, approx. 0.5 μM. This value, in agreement with that already reported for mammalian SHMT (Strong et al., 1989), was calculated assuming that only the pro*S* form of tetrahydrofolate bound to the enzymes. As also shown in Figure 3 and Table 1, the amount of tetrahydrofolate bound per molecule of enzyme indicates that the monomeric T-protein had one binding site for $H_4PteGlu_5$, whereas the tetrameric SHMT had four. To determine the dissociation constant of CH_2-$H_4PteGlu_5$, ^{3}H-labelled CH_2-$H_4PteGlu_5$ was first obtained by incubating various concentrations of $^3H_4PteGlu_5$ in the presence of 1 mM formaldehyde. In our experimental conditions, and for all the concentrations used, the conversion of $H_4PteGlu_5$ into CH_2-$H_4PteGlu_5$ was complete in 5 min, as indicated by the change in A_{290} (Kallen and Jencks, 1966) (results not shown), and purified enzymes were added thereafter. Our results indicate that the dissociation constants obtained with CH_2-$H_4PteGlu_5$ were higher than with $H_4PteGlu_5$. Indeed, the values measured with CH_2-$H_4PteGlu_5$ were 2.7 μM for the T-protein and 7 μM for SHMT (Table 1). Alternatively, ^{3}H-labelled CH_2-$H_4PteGlu_5$ could be obtained by incubating $^3H_4PteGlu_5$ in the presence of SHMT and 5 mM serine. Under these experimental conditions, the dissociation constant of CH_2-$H_4PteGlu_5$ for SHMT was 5 μM, a value roughly comparable with the 7 μM found with the previous method (Table 1).

Our results indicate that T-protein and SHMT have roughly similar dissociation constants for both $H_4PteGlu_5$ and CH_2-$H_4PteGlu_5$, which raises the question of the coupling of the reactions catalysed by these two enzymes. Indeed, the operation of the overall glycine-cleavage system in green leaf mitochondria requires rapid recycling of CH_2-$H_4PteGlu_5$ into $H_4PteGlu_5$, i.e. the SHMT reaction must be continuously driven towards the production of serine, the unfavourable direction {$K_{eq.}$ = [glycine] [CH_2-$H_4PteGlu_5$]/[serine] [$H_4PteGlu_5$] = 15 (Besson et al., 1993)}.

Table 1 Dissociation constants of $H_4PteGlu_5$ and CH_2-$H_4PteGlu_5$ for the T- and SHMT proteins

K_d values were calculated assuming that only the pro*S* form of $H_4PteGlu_5$ and the pro*R* form of CH_2-$H_4PteGlu_5$ were recognized by the T and SHMT enzymes. (a) CH_2-$H_4PteGlu_5$ was obtained by incubating various concentrations of $H_4PteGlu_5$ in the presence of 1 mM formaldehyde for 5 min before the addition of proteins. (b) CH_2-$H_4PteGlu_5$ was obtained by incubating various concentrations of $H_4PteGlu_5$ in the presence of 5 mM serine and SHMT. It has previously been shown (Besson et al., 1993) that under these conditions, the large excess of serine rapidly leads to the total conversion of $H_4PteGlu_5$ (pro*S*) into CH_2-$H_4PteGlu_5$ (pro*R*). The enzyme concentrations were 0.8 μM for the T-protein (monomer) and 0.16 μM for SHMT (tetramer). K_d and n (number of binding sites) were determined by Scatchard plots and by computer-fitting the experimental values to the theoretical hyperbolic curve (see the Materials and methods section). The values are means ± S.E.M. of three different determinations.

	$H_4PteGlu_5$		(a) CH_2-$H_4PteGlu_5$		(b) CH_2-$H_4PteGlu_5$	
	K_d (μM)	n	K_d (μM)	n	K_d (μM)	n
T	0.5 ± 0.1	1 ± 0.1	2.7 ± 0.5	0.9 ± 0.1	–	–
SHMT	0.5 ± 0.1	3.7 ± 0.4	7 ± 1	4 ± 0.3	5 ± 1	4.7 ± 0.4

Table 2 Steady-state levels of CH_2-$H_4PteGlu_n$ (n = 1 and 5) during the course of glycine oxidation in a matrix extract

The final concentration of proteins was 0.6 mg/ml. The reaction was initiated by the addition of either 25 μM or 50 μM $H_4PteGlu_n$ (pro*S*). The reaction was at equilibrium in about 2 min (Bourguignon et al., 1988). After 4 min, two samples were withdrawn for CH_2-$H_4PteGlu_n$ determination. The values presented here are the averages of these two measurements. Calculations were made taking into account the fact that only half of the initial $H_4PteGlu_n$, the pro*S* form, participated in the reaction.

Initial concentration of $H_4PteGlu_1$ or $H_4PteGlu_5$ (pro*S*) (μM)	Final concentration			
	CH_2-$H_4PteGlu_1$ (μM)	CH_2-$H_4PteGlu_1$ (% of total)	CH_2-$H_4PteGlu_5$ (μM)	CH_2-$H_4PteGlu_5$ (% of total)
25	21	84	17	68
50	38	76	40	80

Table 3 Maximal activities of the glycine-cleavage system and SHMT in a matrix extract of pea leaf mitochondria

The values are means ± S.E.M. of four different determinations.

iGlycine decarboxylase ı(nmol of NADH/ ımin per mg of protein)	SHMT (nmol of $CH_2H_4PteGlu_5$/ min per mg of protein)
250 ± 50	100 ± 20

A possible function of the polyglutamate chain of folates may be the co-ordination of sequential folate-dependent enzymes (i.e. T and SHMT) by enhancing channelling of intermediates (McGuire and Bertino, 1981; MacKenzie, 1984; Schirch and Strong, 1989). If this is true, folate polyglutamate intermediates will not equilibrate with the bulk solvent but will be directly transferred between the active sites of the different proteins. In order to test this hypothesis we measured the steady-state levels of CH_2-$H_4PteGlu_n$ (n = 1 or 5) produced during the course of glycine oxidation and at various initial concentrations of $H_4PteGlu_5$. As shown in Table 2, [CH_2-$H_4PteGlu_n$] was always high and represented approx. 65–80 % of the total tetrahydrofolate concentration (assuming that only the active pro*S* form of $H_4PteGlu_n$ was used in the reaction). This indicates that, in these *in vitro* experimental conditions, CH_2-$H_4PteGlu_n$ produced in excess is released into the bulk phase and is not apparently channelled through a supramolecular complex (T- and SHMT proteins). As discussed below, the *in vivo* situation might, however, differ because of a much higher protein concentration. On the other hand, the high CH_2-$H_4PteGlu_n$/$H_4PteGlu_n$ ratio observed during glycine oxidation strongly supports the idea that the glycine-cleavage activity overwhelmed SHMT activity. Indeed, a comparison of their maximal rates in a matrix extract of proteins, a situation supposed to reflect the relative activities *in vivo*, indicates (Table 3) that the maximal velocity of the glycine-cleavage system was approx. 2–2.5 times higher than the maximal rate of serine-into-glycine conversion through SHMT. This means that, during the course of glycine oxidation, the conversion of $H_4PteGlu_n$ into CH_2-$H_4PteGlu_n$ occurred principally via T-protein activity rather than SHMT. Therefore the operation of the glycine-cleavage system always maintained a high CH_2-$H_4PteGlu_n$ level, a situation that favoured the backward reaction catalysed by SHMT.

DISCUSSION

Our results indicate that $H_4PteGlu_5$, the predominant tetrahydrofolate form within the matrix space of pea leaf mitochondria, had a higher binding affinity for the T-protein of the glycine-cleavage complex and SHMT (K_d 0.5 μM for both enzymes) than CH_2-$H_4PteGlu_5$ (K_d 2.7 and 7 μM respectively). In leaf mitochondria, the major function of SHMT is to recycle CH_2-$H_4PteGlu_5$, produced by the T-protein activity, to $H_4PteGlu_5$, to allow the continuous operation of the glycine-oxidation reaction. However, the SHMT-catalysed reaction is the critical step of the overall system. Indeed, the binding affinity of CH_2-$H_4PteGlu_5$ for SHMT was lower than that of $H_4PteGlu_5$ and the rate constant of the reaction converting CH_2-$H_4PteGlu_n$ into $H_4PteGlu_n$ was 15 times lower than the rate constant for the reverse reaction (Besson et al., 1993). Clearly, the SHMT reaction must be permanently pushed out of equilibrium, towards the production of serine and $H_4PteGlu_n$, to allow the whole process to take place. This was apparently the case because we observed

high CH_2-$H_4PteGlu_n$/$H_4PteGlu_n$ ratios in the medium during the steady-state course of glycine oxidation. These high ratios were presumably the result of the highest glycine decarboxylase activity, compared with SHMT activity, that we measured in the matrix space. In addition, the high CH_2-$H_4PteGlu_n$ level detected during this reaction strongly suggests that this compound was not channelled between the T-protein and SHMT, as it accumulated in the bulk medium. However, it must be pointed out that our experimental conditions did not really reflect the *in vivo* situation. Indeed the concentration of proteins used in these assays never exceeded 1 mg/ml, a value 400 times lower than that recorded *in vivo* in the matrix space of pea leaf mitochondria (Oliver et al., 1990b). Thus the $H_4PteGlu_n$/protein ratio we used in our *in vitro* experiments was high compared with the situation *in vivo*. In intact mitochondria, enzymes might behave differently, and the very high protein concentration, together with the relatively low tetrahydrofolate level, could lead to a situation where folate compounds are not released into the bulk medium. Indeed, as discussed below, there are apparently more binding sites for folate in the matrix space of mitochondria than folate compounds (Besson et al., 1993).

Our results indicate that binding of $H_4PteGlu_n$ to the glycine decarboxylase complex and SHMT increase with increasing number of glutamates up to six residues. From these data it is tempting to postulate that binding of $H_4PteGlu_n$ correlated with the number of negatively charged α-COO^- groups of the poly-γ-glutamate chain. Indeed, it has been reported that basic groups on proteins, such as arginine for sheep liver SHMT (Usha et al., 1992) and lysine for thymidylate synthetase (Maley et al., 1982), are implicated in the binding of folylpolyglutamates. In this connection, it was recently shown that the T-protein of the glycine-cleavage system from pea leaf mitochondria is characterized by the presence of aligned basic amino acids (Bourguignon et al., 1993), a favourable condition for ionic interaction with the negatively charged polyglutamate chain. However, a close comparison of the known primary amino acid sequence of the T-protein (Bourguignon et al., 1993) with that of SHMT (Turner et al., 1992), both enzymes originating from pea leaf mitochondria, did not reveal conserved folylpolyglutamate-binding consensus sequences (Bourguignon et al., 1993). Each folate-dependent enzyme might have its own specific folate-binding site, or the binding sites might rely on secondary or tertiary structures.

Interestingly, our results did not show a strict relationship, with regard to number of glutamates, between binding affinity ($6 > 5 > 4 > 3 > 2 > 1$) and catalytic efficiency ($6 \approx 5 \approx 4 \geqslant 3 > 2 > 1$) of $H_4PteGlu_n$. Similar results have also been obtained with formiminotransferase-cyclodeaminase (Paquin et al., 1985), and one may question the physiological necessity for glutamate chains with more than three residues. It has been proposed that longer polyglutamate chains might play an important role in channelling. Supporting this view, it has been shown with formiminotransferase-cyclodeaminase (Paquin et al., 1985) that the specificity for channelling was optimum for $H_4PteGlu_5$, one of the most predominant folylpolyglutamate species in animals (Kisliuk, 1981) and plants (Imeson et al., 1990; Besson et al., 1993). The optimal glutamate chain length must be a compromise between a strong affinity for folate-dependent enzymes, which limits diffusion into the bulk medium, and the ability of the molecule to move from one catalytic site to another. Although channelling was not evident with the enzyme systems studied here, our results indicate that $H_4PteGlu_n$ binding to folate-dependent proteins significantly contributed to the protection of this readily oxidizable compound [for *in vitro* manipulation this molecule requires the presence of high concentrations of reducing agents or strict anaerobic conditions (Bourguignon et al., 1988)]. As the matrix space of mitochondria is obviously not anaerobic, we are forced to conclude that *in vivo* $H_4PteGlu_n$ is protected from O_2, at least partially, by association with folate-dependent proteins. In support of this suggestion, and taking into account the fact that the tetrameric SHMT bound one folate molecule per subunit and the monomeric T-protein bound one folate molecule, it was previously calculated that these two proteins were able to bind up to twice the folate content of the mitochondria (Besson et al., 1993). This high mitochondrial concentration of folate-binding sites should considerably reduce the tetrahydrofolate diffusional pathway in the matrix space.

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