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Resolution and characterization of the glycine-cleavage reaction in pea leaf mitochondria

Properties of the forward reaction catalysed by glycine decarboxylase and serine hydroxymethyltransferase

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High-molecular-mass proteins from pea (*Pisum sativum*) mitochondrial matrix retained on an XM-300 Diaflo membrane ('matrix extract') exhibited high rates of glycine oxidation in the presence of NAD^+ and tetrahydropteroyl-L-glutamic acid (H_4folate) as long as the medium exhibited a low ionic strength. Serine hydroxymethyltransferase (SHMT) (4×53 kDa) and the four proteins of the glycine-cleavage system, including a pyridoxal phosphate-containing enzyme ('P-protein'; 2×97 kDa), a carrier protein containing covalently bound lipoic acid ('H-protein'; 15.5 kDa), a protein exhibiting lipoamide dehydrogenase activity ('L-protein'; 2×61 kDa) and an H_4folate-dependent enzyme ('T-protein'; 45 kDa) have been purified to apparent homogeneity from the matrix extract by using gel filtration, ion-exchange and phenyl-Superose fast protein liquid chromatography. Gel filtration on Sephacryl S-300 in the presence of 50 mM-KCl proved to be the key step in disrupting this complex. During the course of glycine oxidation catalysed by the matrix extract a steady-state equilibrium in the production and utilization of 5,10-methylene-H_4folate was reached, suggesting that glycine cleavage and SHMT are linked together via a soluble pool of H_4folate. The rate of glycine oxidation catalysed by the matrix extract was sensitive to the NADH/NAD^+ molar ratios, because NADH competitively inhibited the reaction catalysed by lipoamide dehydrogenase.

INTRODUCTION

During photorespiration in the leaves of higher plants having the 'C_3' pathway of photosynthesis, glycine is rapidly oxidatively decarboxylated in the mitochondria to yield CO_2, NH_3, serine and NADH (Lorimer & Andrews, 1981; Tolbert, 1980). The available evidence suggests that, in green-leaf cells, the complete process occurs within the mitochondrial matrix via a co-ordinated sequence of reactions (Keys, 1980). Glycine is cleaved in the matrix space (Douce, 1985) by the glycine-cleavage system (aminomethyltransferase, EC 2.1.2.10) to CO_2, NH_3 and 5,10-methylene-5,6,7,8-tetrahydropteroyl-L-glutamic acid (5,10-CH_2H_4folate). The latter compound produced reacts with a second molecule of glycine to form serine in a reaction catalysed by serine hydroxymethyltransferase (EC 2.1.2.1; SHMT) (Schirch, 1982; Rao & Rao, 1982). The glycine-cleavage system has been purified from plants (Walker & Oliver, 1986*a*), animals (Kikuchi & Hiraga, 1982) and bacteria (Klein & Sagers, 1966, 1967; Kochi & Kikuchi, 1974) and consists of four protein components (Klein & Sagers, 1967; Kikuchi & Hiraga, 1982), which have been tentatively named 'P-protein' (a pyridoxal phosphate-containing protein), H-protein (a lipoic acid-containing protein, T-protein (a protein catalysing the tetrahydrofolate-dependent step of the reaction) and L-protein (a lipoamide dehydrogenase). The glycine decarboxylase is present in low amounts in etiolated pea leaves, but increases dramatically upon light exposure (Day *et al.*, 1985; Walker & Oliver, 1986*b*). In addition this enzyme has been successfully solubilized from pea leaf mitochondria as an acetone-dried powder (Sarojini & Oliver, 1983; Walker & Oliver, 1986*a*). Glycine decarboxylase thus prepared could catalyse the reverse of the glycine decarboxylase reaction by converting serine, NH_4^+ and CO_2 into glycine. However, no attempt has been made to monitor the forward reaction, that is, the conversion of glycine into serine and NADH in the presence of SHMT. The studies of Walker & Oliver (1986*a*) indicated that precipitation of the enzyme from pea leaf mitochondria by cold acetone produced a crude extract exhibiting glycine–bicarbonate exchange. Under these conditions, the P-, H- and T-proteins can be separated by $(NH_4)_2SO_4$ and poly(ethylene glycol) precipitations, gel filtration and ion-exchange chromatography (Walker & Oliver, 1986*a*). However, the isolation of SHMT was not carried out, and the L-protein was not purified but was studied immunochemically. The results of Walker & Oliver (1986*a*) therefore suggest that, after acetone extraction, the glycine decarboxylase multienzyme complex exists in solution predominantly as four separate enzymes.

Fairly recently a fully active glycine-cleavage system associated with SHMT has been isolated from the matrix

Abbreviations used: H_4folate, L-5,6,7,8-tetrahydropteroyl-L-glutamic acid; P-, L-, H- and T-proteins are defined in the text; SHMT, serine hydroxymethyltransferase; 5,10-CH_2-, 5,10-methylene-; H_2folate, L-7,8-dihydropteroyl-L-glutamic acid; f.p.l.c. (Pharmacia FPLC), fast protein liquid chromatography; LDS, lithium dodecyl sulphate.

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of pea leaf mitochondria (Neuburger *et al.*, 1986). The physiological integrity of this complex was maintained if the preparation of the enzymic extract was carried out at low ion concentrations and at about neutral pH in order to prevent the release of the H-protein from the glycine decarboxylase (Kikuchi & Hiraga, 1982). With the aim of further clarifying the mechanisms of glycine oxidation in green leaf mitochondria, the present paper details the rapid isolation of all the protein components involved in glycine oxidation coupled to serine production. In addition, we here report some regulatory properties of this multienzyme complex.

MATERIAL AND METHODS

Isolation of mitochondria

Mitochondria were isolated and purified from pea leaves as described by Douce *et al.* (1987), using self-generating Percoll gradients and a linear gradient of 0–10% (w/v) polyvinylpyrrolidone 25 (top to bottom). The mitochondria were found in a tight white band near the bottom of the tube, whereas the thylakoids remained near the top of the tube. The mitochondria were subsequently concentrated by differential centrifugation. The purified mitochondria were suspended in a medium (suspending medium) containing 0.3 M-mannitol, 10 mM-phosphate buffer, pH 7.2, 1 mM-EDTA and 1 mM-β-mercaptoethanol at approx. 50–100 mg of protein/ml. O_2 uptake was measured at 25 °C with a Clark-type oxygen electrode purchased from Hansatech (King's Lynn, Norfolk, U.K.). The reaction medium contained 0.3 M-mannitol, 5 mM-$MgCl_2$, 10 mM-KCl, 10 mM-phosphate buffer (pH 7.2), 0.1% (w/v) defatted bovine serum albumin and known quantities of mitochondrial protein in a total volume of 1 ml. With 10 mM-glycine as substrate, the respiratory-control index was routinely better than 4 and the State-3 rate of O_2 uptake was higher than 100 nmol/min per mg of protein (Douce *et al.*, 1987). The mitochondria were better than 95% intact as judged by their impermeability to cytochrome *c* (Douce *et al.*, 1972).

Solubilization of the glycine-cleavage system and SHMT from pea leaf mitochondria

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 μM-pyridoxal phosphate, 1 mM-serine, 1 mM-octyl β-D-glucopyranoside (Calbiochem), pH 7.0. The addition of 4 μM-leupeptin to the lysis buffer provides further protection of the enzymes against endogenous proteinases. Total release of the matrix protein was achieved by three cycles of freezing and thawing (Neuburger *et al.*, 1986). The mitochondrial suspension was frozen by placing it at liquid-N_2 temperature for 2 min. The frozen mitochondrial suspension was then maintained at 30 °C until thawed. This procedure breaks about 98% of the mitochondria. The suspension of broken mitochondria was centrifuged at 100000 *g* for 2 h (36000 rev./min in a Beckman SW-40 rotor) to remove all the mitochondrial membranes. The high-molecular-mass complexes (above 300 kDa) from the matrix fraction were obtained by filtration of the supernatant on a Diaflo membrane XM-300 using a stirred cell on a magnetic-stirring table (Amicon). The flow rate was 1 ml/min. All the proteins involved in the conversion of glycine into serine were retained by this selectively retentive membrane (matrix extract, final volume 2 ml) as long as the pH is maintained at about neutrality and the medium exhibits a low ionic strength. Under these conditions we have verified that the small protein component of the glycine-cleavage system ('H-protein'; 15.5 kDa), as well as SHMT, did not pass through the Diaflo membrane. At this stage, the glycine-cleavage/SHMT system could be stored at −80 °C under N_2 without deterioration for many months. However, after several cycles of freezing and thawing the glycine-cleavage complex lost part of its activity, owing to P-protein denaturation.

Assay of glycine oxidation in matrix extract

Glycine oxidation was assayed at 25 °C by measuring the formation of NADH or serine that was dependent upon the presence of both glycine and H_4folate (L-5,6,7,8-tetrahydropteroyl-L-glutamic acid) (Cossins, 1987). The standard reaction mixture contained, in a total volume of 400 μl: 5 mM-Mops, 5 mM-Tris (pH 7.2), 1 mM-$MgCl_2$, 1 mM-EGTA, 20 μM-pyridoxal phosphate, 1 mM-β-mercaptoethanol, 2 mM-NAD^+, 5 μg of antimycin A, 0.35 mM-H_4folate [H_4folate had been prepared chemically by the catalytic hydrogenation of L-pteroylglutamic acid according to the method of Huennekens *et al.* (1963)] and known quantities of matrix extract or proteins isolated from the matrix extract. The medium and all the stock solutions were bubbled with argon [in the presence of atmospheric O_2 there was a rapid oxidation of H_4folate to H_2folate (L-7,8-dihydropteroyl-L-glutamic acid), which has an absorption maximum at 282 nm]. When H_4folate is freshly dissolved, the absorption maximum is at 298 nm, but within 10 min, in the presence of O_2, the spectrum shifts to 282 nm (Hatefi *et al.*, 1959). The reaction, carried out under strict anaerobic conditions, was initiated by addition of 20 mM-glycine, unless otherwise stated. The rate of NADH formation was measured at 340 nm using a Kontron (Uvikon 810) spectrophotometer. At various times, 50 μl aliquots were taken and added to 50 μl of cold 1 M-HCl. The samples were centrifuged for 5 min at 10000 *g* (Beckman Microfuge B) to remove proteins. The supernatant was used for serine determination. Serine was analysed in sodium citrate buffers with an amino acid analyser (model 118C; Beckman Instruments, Palo Alto, CA, U.S.A.).

Assay of serine hydroxymethyltransferase

The rate of the reaction was monitored by trapping the formaldehyde product isolated from 5,10-CH_2-H_4folate as the dimedon complex and extracting it into a layer of toluene (Schirch, 1984).

The assay mixture contained 20 mM-potassium phosphate, pH 7.4, 1 mM-β-mercaptoethanol, 2 mM-dithiothreitol, 1 mM-EDTA, 4 mM-L-[3-^{14}C]serine (2.4×10^4 Bq/nmol) and enzyme in a total volume of 0.2 ml. All components were first incubated for 5 min at 37° under argon. The reaction was then initiated by the addition of 2 mM-H_4folate and was terminated with 0.4 M-sodium acetate (pH 4.5), 20 mM-formaldehyde and 0.1 M-dimedon (added in succession). The mixture was heated for 5 min at 100 °C to accelerate the formation of the [^{14}C]formaldehyde–dimedon derivative. The tubes were then cooled for 5 min in an ice bath before the dimedon derivative was extracted by vigorous shaking

with 3 ml of toluene (Taylor & Weissbach, 1965). After centrifugation at 2000 rev./min for 10 min (Kontron SS34 rotor), 2 ml of upper phase were removed for radioactivity counting in an Intertechnique SL 4000 liquid-scintillation spectrometer.

Assay of P-, H- and T-proteins

The activities of P- and H-proteins were determined by measuring the amounts of [^{14}C]bicarbonate fixed to the carboxy-group carbon atom of glycine during the exchange reaction (Hiraga & Kikuchi, 1980). The assay mixture, in a final volume of 200 μl, contained 25 mM-potassium phosphate, pH 7.0, 1 mM-dithiothreitol, 20 μM-pyridoxal phosphate, 25 mM-glycine and 40 mM-[^{14}C]bicarbonate (500 Bq/nmol), 30 μg of H-protein for P-protein assay or 30 μg of P-protein for H-protein assay, and an appropriate amount of the enzyme preparation to be tested. The reaction was initiated by addition of bicarbonate. At 5 and 10 min of incubation, a portion (200 μl) of the reaction mixture was transferred to a glass radioactivity-counting vial containing 80 μl of acetic acid and the mixture was dried on a hot plate. The radioactivity was determined with an Intertechnique SL 4000 spectrometer after addition of 10 ml of aqueous counting scintillant. A unit of P-protein (in the presence of saturating amount of H-protein) was defined as the amount of P-protein that catalysed the fixation of 1 μmol of $^{14}CO_2$ into glycine/min. Likewise 1 unit of H-protein (in the presence of saturating amount of P-protein) was also defined as the amount of H-protein that catalysed the fixation of 1 μmol of $^{14}CO_2$ into glycine/min.

The activity of T-protein was assayed by measuring the formation of NADH that was dependent upon the presence of saturating amounts of P-, H- and L-proteins and SHMT. The assay mixture contained, in a total volume of 400 μl, 5 mM-Mops, 5 mM-Tris, pH 7.3, 1 mM-β-mercaptoethanol, 1 mM-$MgCl_2$, 1 mM-EDTA, 20 μM-pyridoxal phosphate, 0.35 mM-H_4folate, 20 μg of P-protein, 20 μg of H-protein, 20 μg of L-protein and 40 μg of SHMT. The reaction was initiated by the addition of an appropriate amount of the enzyme preparation (T-protein) to be tested. The rate of NADH formation was measured under anaerobiosis at 340 nm by using a Kontron (Uvikon 810) spectrophotometer. T-protein activity was expressed as μmol of NADH formed/min.

Assay of L-protein

The activity of L-protein was assayed by measuring the formation of NADH that was dependent upon the presence of dihydrolipoic acid. The standard reaction mixture contained 5 mM-Mops, 5 mM-Tris, pH 7, 1 mM-β-mercaptoethanol, 1 mM-EGTA, 2 mM-NAD^+ and 2 mM-dihydrolipoate and enzyme (L-protein) in a total volume of 400 μl. The reaction, carried out under strict anaerobic conditions, was initiated by addition of dihydrolipoate. The rate of NADH formation was measured at 340 nm by using a Kontron (Uvikon 810) spectrophotometer. Dihydrolipoic acid was prepared from lipoic acid (DL-6,8-thioctic acid; Sigma) as described by Kochi & Kikuchi (1976).

Assay of pyruvate dehydrogenase

The pyruvate dehydrogenase complex was assayed at 340 nm by using a Kontron (Uvikon 810) spectrophotometer. The standard assay mixture contained 50 mM-Mops, pH 7.5, 0.2 mM-thiamin pyrophosphate, 1 mM-$MgCl_2$, 2 mM-NAD^+, 0.1 mM-CoASH, 2.5 mM-cysteine hydrochloride, 1 mM-potassium pyruvate and enzyme in a total volume of 1 ml (Rapp *et al.*, 1987).

Purification of P-, L-, T- and H-proteins and SHMT from matrix extract

Step 1: matrix-extract fractionation. The matrix extract (2 ml; 80 mg of protein) supplemented with 4 μM-leupeptin was applied to a 2.5 cm × 35 cm column of Sephacryl S-300 (Superfine grade; Pharmacia) equilibrated in 50 mM-KCl/5 mM-Mops/5 mM-Tris(pH 7.5)/2 mM-β-mercaptoethanol/1 mM-EGTA/1 mM-serine/1 mM-glycine (Buffer A). The column connected to 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 and SHMT were eluted together (heavy fraction), as were T- and H-protein (light fraction), and L-protein activity was eluted between light and heavy fractions (intermediary fraction). The heavy and L-protein fractions were combined separately and concentrated to 1–2 ml by ultrafiltration (XM-50 membrane; Amicon). Pooled fractions containing H- and T-protein activities (light fraction) were dialysed and concentrated to 1–2 ml by ultrafiltration (PM 10 membrane; Amicon) in 5 mM-Mops/5 mM-Tris (pH 7.5)/2 mM-β-mercaptoethanol/1 mM-EGTA (buffer D).

Step 2: purification of P-protein and SHMT from heavy fraction. A portion of the heavy fraction (see Step 1) (19 mg of protein) was applied to a Trisacryl M DEAE column (Industrie Biologique Française; 1 cm × 41 cm) designated to fit into Pharmacia's f.p.l.c. system and previously equilibrated with buffer A containing 10 mM-phosphate buffer, pH 7.5, instead of Tris and Mops buffers. P-protein and SHMT were eluted at 4 °C with a continuously increasing KCl gradient (50–1000 mM) in the same buffer (flow rate 0.5 ml/min; fraction size 2 ml). SHMT activity was eluted in the void volume. By contrast, P-protein activity emerged as a very large peak at 100 mM-KCl. Fractions containing SHMT and P-protein were concentrated by ultrafiltration through a Diaflo membrane XM-100 (Amicon). The pooled fractions of P-protein were then applied to a phenyl-Superose HR 5/5 column (Pharmacia; 0.5 cm × 5 cm) previously equilibrated with buffer B containing 10 mM-potassium phosphate, pH 7.5, 1 mM-EGTA, 1 mM-β-mercaptoethanol, 1 mM-glycine and 1 M-$(NH_4)_2SO_4$. The column, connected to a Pharmacia f.p.l.c. system, was eluted with a decreasing $(NH_4)_2SO_4$ gradient (1–0 M) (flow rate 0.5 ml · min^{-1}; fraction size 1 ml). P-protein emerged as a peak at 250 mM-$(NH_4)_2SO_4$.

The pooled fractions of SHMT were applied to a mono Q HR 5/5 column (Pharmacia; 5 cm × 5 cm) previously equilibrated with buffer C containing 10 mM-potassium phosphate, pH 6.8, 1 mM-EGTA, 1 mM-β-mercaptoethanol and 1 mM-serine. The column, connected to a Pharmacia f.p.l.c. system, was eluted with a continuously increasing potassium phosphate gradient (10–200 mM) (flow rate 0.3 ml · min^{-1}; fraction size 0.9 ml). SHMT emerged as a sharp peak at 100 mM-potassium phosphate. The fractions containing proteins were combined separately, and each was concentrated to 0.5 ml by use of ultrafiltration on a XM-100 membrane or by use of a fast desalting HR 10/10 f.p.l.c.. Purified P-protein and SHMT were stored at −80 °C in buffer B without $(NH_4)_2SO_4$ or buffer C.

Step 3: purification of H- and T-proteins from light fraction. A portion of the light fraction (see Step 1) (6 mg of protein) was applied to a mono Q HR 5/5 column previously equilibrated in buffer D. H- and T-protein were eluted at 4 °C and at pH 7.5 with a continuously increasing potassium phosphate gradient (0–500 mM) in buffer D (flow rate 0.3 ml · min^{-1}; fraction size 0.9 ml). T-protein, which is positively charged at physiological pH, was eluted in the void volume; by contrast, H-protein emerged as a sharp peak at 210 mM-potassium phosphate. Purified H- and T-protein fractions concentrated to 0.5 ml by use of ultrafiltration on a YM-10 membrane were stored at −80 °C in buffer D.

Step 4: purification of L-protein from intermediary fraction. L-protein activity was eluted between the light and heavy fractions (see Step 1) (12 mg of protein) was applied to a mono Q HR 5/5 column previously equilibrated in 10 mM-potassium phosphate, pH 7.5. L-protein was eluted at 4 °C and at pH 7.5 with a continuously increasing potassium phosphate gradient (10–500 mM) (flow rate 0.3 ml · min^{-1}; fraction size, 0.9 ml). L-protein emerged as a single peak at 270 mM-potassium phosphate.

Electrophoresis

(a) LDS/polyacrylamide-gel electrophoresis. The electrophoresis was performed at 4 °C in LDS/polyacrylamide slab gels containing a 7.5–15% or 10–15% linear acrylamide gradient. The experimental conditions for gel preparation, sample solubilization, electrophoresis and gel staining were detailed by Chua (1980).

(*b*) **Native gels.** Gels consisting of 5% acrylamide and 0.2% bisacrylamide in 40 mM-Tris/HCl, pH 8.0, were prepared as described by Laemmli (1970), except that detergent was omitted from the different buffers. Electrophoresis was performed at 15 mA/gel at 4 °C for 6 h.

M_r determination

The M_r values for the proteins were determined by gel filtration (Andrews, 1965) by using Ultrogel AcA-34 (gel-filtration range 20000–350000 M_r; IBF) equilibrated in buffer A. Protein standards (Pharmacia calibration kit) were ferritin (molecular mass 440 kDa), bovine liver catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa) and cytochrome *c* (12.5 kDa). The M_r was calculated from a plot of log M_r against elution volume.

RESULTS

Protein components of the glycine decarboxylase system and SHMT

The isolation method described in the Materials and methods section provides all the protein components of the glycine decarboxylase and SHMT in good yield and at a high level of purity in 2 working days. Gel filtration on Sephacryl S-300 in the presence of 50 mM-KCl and at pH 7.5 proved to be the key step in disrupting the 'multienzyme complex' obtained by filtration of the matrix proteins on a Diaflo membrane XM300 and involved in glycine oxidation (Neuburger *et al.*, 1986). A

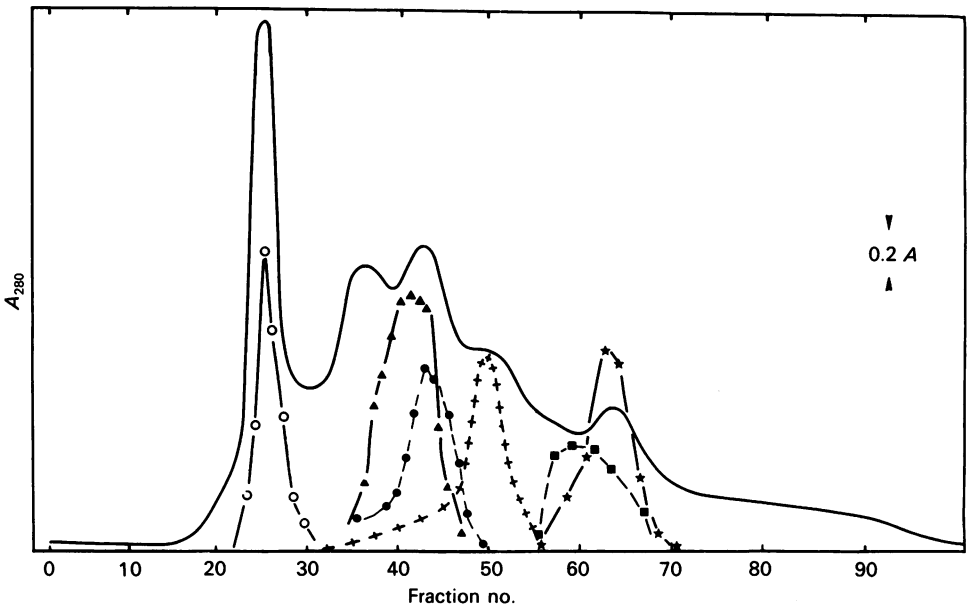

Fig. 1. Separation of pyruvate dehydrogenase, glycine-cleavage proteins (P-, H-, T- and L-proteins) and SHMT from matrix extract by gel filtration through Sephacryl S-300

Approx. 2 ml (80 mg) of the matrix extract, which was obtained by filtration of the matrix proteins on a Diaflo membrane XM 300 (see the Materials and methods section), was applied to the Sephacryl S-300 column (2.5 cm × 35 cm; flow rate approx. 0.5 ml · min^{-1}) equilibrated in 50 mM-KCl. Fractions (about 2 ml each) were collected. The pyruvate dehydrogenase activity at the top of the peak 5.5 nkat · ml^{-1}; the P-, H-, T- and L-protein activities at the top peaks were 5.1, 4.3, 6.5 and 28 nkat · ml^{-1} respectively; and the SHMT activity at the top of the peak was 5.3 nkat · ml^{-1}. Heavy fraction comprised P-protein + SHMT, the light fraction H- + T-proteins and the intermediary fraction L-protein. Key to symbols: ○, pyruvate dehydrogenase; ▲, ●, heavy fraction (▲, SHMT; ●, P-protein); ■, ★, light fraction (■, T-protein; ★, H-protein); +, intermediary fraction (L-protein); ——, A_{280}.

typical elution profile is shown in Fig. 1. Under these conditions, all the proteins (P, H, T and L) associated with the glycine decarboxylase were clearly resolved. However, P-protein and SHMT, which exhibit the same M_r, were eluted almost together (heavy fraction). Interestingly, the pyruvate dehydrogenase complex, which is more stable, was eluted in the void volume (Fig. 1). When the same experiment was carried out at low ionic strength and at a pH of about neutrality, a considerable amount of H-protein was eluted in association with P-protein (results not shown; see also Kikuchi & Hiraga, 1982).

Purification of P-protein and SHMT. The overall scheme and results of the purification procedure for P-protein and SHMT are shown in Table 1. The procedure resulted in a 9-fold purification for P-protein and 59-fold purification for SHMT. After the subsequent gel-filtration and ion-exchange-chromatography steps, P-protein and SHMT were almost pure, with molecular masses of 97 ± 2 kDa and 53 ± 2 kDa respectively, as determined by LDS/polyacrylamide-gel electrophoresis (Fig. 2). The molecular masses of P-protein and SHMT were estimated to be 210 ± 7 kDa and 220 ± 10 kDa by using a calibrated Ultrogel AcA-34 gel column (Andrews, 1965) and polyacrylamide-gel electrophoresis under non-denaturating conditions. This would therefore suggest that the native P-protein was a dimer composed of two identical subunits of molecular mass 97 kDa (Walker & Oliver, 1986*a*; Hiraga & Kikuchi, 1980) and the native SHMT was a tetramer composed of four identical subunits of molecular mass 53 kDa (Schirch & Peterson, 1980; Rao & Rao, 1982).

Purification of H-protein and T-protein. A summary of the purification scheme for the H- and T-protein is

Table 1. Purification of P-protein (P-prot.) and SHMT from pea leaf mitochondria

	Total protein (mg)		Total activity (nkat)		Specific activity (nkat · mg^{-1})		Yield (%)	
Purification stage	P-prot.	SHMT	P-prot.	SHMT	P-prot.	SHMT	P-prot.	SHMT
Matrix fraction	109.8		87.8	98.7	0.80	0.9	100	100
Matrix extract	80		87.7	97.3	1.09	1.2	100	98.5
Sephacryl S-300 pool	23.2		61	80.8	2.6	3.5	69	82
Trisacryl M DEAE pool	6.5	3.1	33	75	5.1	23.8	37.6	76
Phenyl-Superose HR 5/5 pool (P-prot.) or Mono Q HR 5/5 pool (SHMT)	3.8	1.4	27.7	72	7.3	53.1	31.5	72.5

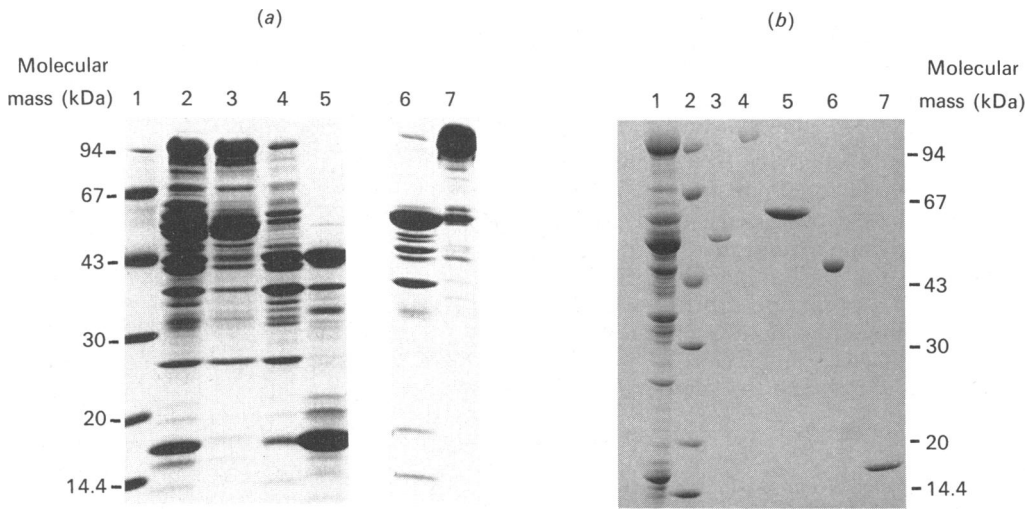

Fig. 2. Electrophoretic analysis of all protein components of glycine decarboxylase and SHMT

(*a*) Proteins were separated on a SDS/7.5–15% gradient polyacrylamide slab gel stained with Coomassie Brilliant Blue R-250. Lane 1, Pharmacia standards, 15 μg; 2, matrix extract, 60 μg; 3, heavy fraction (see Fig. 2) containing the P-protein and SHMT, 50 μg; 4, intermediary fraction (see Fig. 2) containing the L-protein, 50 μg; 5, light fraction (see Fig. 2) containing the T- and H- protein, 50 μg; 6, trisacryl M DEAE pool containing SHMT (see Table 1), 25 μg; 7, trisacryl M DEAE pool (see Table 1) containing P-protein, 25 μg. (*b*) Proteins were separated on a SDS/10–15% gradient polyacrylamide slab gel. Lane 1, matrix extract, 60 μg; 2, Pharmacia standards, 15 μg; 3, purified SHMT (53 kDa), 4 μg; 4, purified P-protein (97 kDa), 3 μg; 5, purified L-protein (61 kDa), 7.5 μg; 6, purified T-protein (43 kDa), 6 μg; 7, purified H-protein (15.5 kDa), 6 μg (see Table 1).

Table 2. Purification of H-protein (H-prot.) and T-protein (T-prot.) from pea leaf mitochondria

Purification stage	Total protein (mg)		Total activity (nkat)		Specific activity (nkat · mg^{-1})		Yield (%)	
	H-prot.	T-prot.	H-prot.	T-prot.	H-prot.	T-prot.	H-prot.	T-prot.
Matrix fraction	109.8		87.8	232	0.80	2.1	100	100
Matrix extract	80		87.7	231	1.09	2.9	100	99.5
Sephacryl S-300 pool	12		46.8	72	3.9	6	53	31
Mono Q HR 5/5 pool	4.4	3.7	33.9	67	7.7	18	38.6	29

shown in Table 2. The procedure resulted in a 9.6-fold purification for H-protein and 8.8-fold for T-protein. LDS/polyacrylamide-gel electrophoresis of purified H- and T-proteins resulted in single bands corresponding to a molecular mass of $\sim 15.5 \pm 1$ kDa for H-protein and of 45 ± 2 kDa for T-protein (Fig. 2). The molecular mass of H-protein was estimated to be 15.5 ± 1 kDa by using polyacrylamide-gel electrophoresis under non-denaturating conditions. Since T-protein is positively charged (Okamura-Ikeda *et al.*, 1987), its molecular mass was estimated to be 45 ± 2 kDa by using a calibrated Ultragel AcA-34 gel column. These results strongly suggest that both proteins are composed of a single polypeptide (Walker & Oliver, 1986*a*).

Purification of L-protein. The overall scheme and results of the purification procedure for L-protein (lipoamide dehydrogenase) are shown in Table 3. The procedure resulted in a 50-fold purification. On the basis of LDS/polyacrylamide-gel electrophoresis, the L-protein that emerges from the ion-exchange column was more than 95% pure and exhibited a subunit molecular mass of 61 ± 3 kDa (Fig. 2). The molecular mass of the native enzyme was 120 ± 5 kDa upon gel filtration. From these results the L-protein from pea leaf mitochondria appears to consist of two identical subunits.

Biochemical characterization of the glycine-cleavage system and SHMT

High-molecular-mass proteins from the mitochondrial matrix retained on an XM-300 Diaflo membrane (see the Materials and methods section) produced a matrix extract exhibiting high rates of glycine oxidation in the presence of H_4folate and pyridoxal phosphate (Fig. 3). The apparent Michaelis constants for the substrates glycine and NAD^+ and for pyridoxal phosphate were calculated to be 6.5 mM, 75 μM and 2 μM respectively. This matrix extract exhibited no conspicuous pH-dependence between 6.8 and 7.5. We have verified that the smaller proteins of this complex [H-protein (15.5 kDa) and T-protein (45 kDa)] (see Fig. 2) did not pass through the XM300 Diaflo membrane, suggesting that, at low ionic strength and at a pH of about neutrality, all the proteins of the glycine-cleavage system exist in the form of an enzyme complex which is easily disrupted (Fig. 1). Furthermore the addition of purified H-protein or T-protein to the enzymic extract in the presence of saturating amounts of H_4folate did not significantly change the rate of glycine oxidation (Table 4). These results demonstrate, therefore, that T-protein and H-protein, which acts as a co-substrate with glycine during the decarboxylation catalysed by P-protein (Fujiwara & Motokawa, 1983) were present in saturating amounts in the matrix extract.

We have shown previously that the rate of glycine oxidation catalysed by the matrix extract was clearly

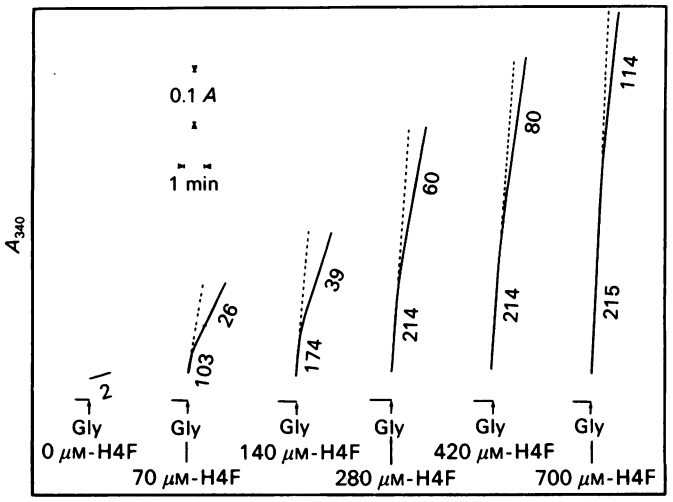

Fig. 3. Effect of H_4folate (H4F) on glycine (Gly) oxidation catalysed by a matrix extract isolated from pea leaf mitochondria

The preparation of the matrix extract is described in the text. The standard reaction mixture (see the text) contained 0.28 mg of matrix extract protein. Glycine oxidation was assayed at 25 °C by measuring the formation of NADH at 340 nm with a Kontron (Uvikon-810) spectrophotometer. The values along the traces refer to nmol of NADH formed/min per mg of protein. Note that, especially at low H_4folate concentrations, the rate of glycine oxidation was clearly biphasic.

Table 3. Purification of L-protein from pea leaf mitochondria

Purification stage	Total protein (mg)	Total activity (nkat)	Specific activity (nkat · mg^{-1})	Yield (%)
Matrix fraction	109.8	517	4.7	100
Matrix extract	80	500	6.3	97
Sephacryl S-300 pool	16.8	363	21.6	70
Mono Q HR 5/5 pool	1.4	325	232	60

Table 4. Effect of P-, H-, T- and L-proteins on glycine oxidation catalysed by matrix extract isolated from pea leaf mitochondria

Complete system contained, in a final volume of 400 μl, 5 mM-Mops, 5 mM-Tris (pH 7.2), 1 mM-β-mercaptoethanol, 1 mM-EGTA, 20 μM-pyridoxal phosphate, 0.35 mM-H_4-folate, 2 mM-NAD^+, 1 mM-$MgCl_2$, 5 μg of antimycin A and 0.30 mg of matrix extract. Purified P-, H-, L- and T-proteins were prepared as described in the Materials and methods section. Reactions were performed and quantified also as described in that section.

System	NADH formed (nmol · min^{-1} · mg of $protein^{-1}$) Initial slope	Final slope
Complete system	173	63
+ P-protein (35 μg)	174	64
+ H-protein (15 μg)	173	63
+ L-protein (25 μg)	173	63
+ T-protein (20 μg)	173	64

biphasic (Neuburger *et al.*, 1986). At low H_4folate concentrations the change of slope was pronounced, whereas at high H_4F concentrations it was hardly visible (Fig. 3). Interestingly, when the NADH concentration attained at the change of slope was plotted against the amount of H_4folate initially added, a straight line was obtained (results not shown), suggesting that this unexpected phenomenon was attributable to partial exhaustion of H_4folate for glycine decarboxylase. In other words this would suggest that, in this particular condition, the rate-limiting step of glycine oxidation after the change of slope may be the regeneration of H_4-folate from 5,10-CH_2-H_4folate by SHMT for glycine decarboxylase functioning. In support of this suggestion, Fig. 4, showing the accumulation of NADH and 5,10-CH_2-H_4folate during the course of glycine oxidation, indicates that 5,10-CH_2-H_4folate accumulated very rapidly up to the change of slope in the rate of NADH production. From this point a steady-state equilibrium in the production and utilization of 5,10-CH_2-H_4folate was reached, and NADH and serine were formed equally (Neuburger *et al.*, 1986), indicating that the glycine-cleavage system and SHMT operate in a concerted manner. The concentration of 5,10-CH_2-H_4folate attained at the equilibrium was dependent upon several critical factors, including the initial concentration of H_4-folate (Fig. 5) and the respective strengths of glycine cleavage and SHMT (results not shown). For example, addition of a large excess of purified SHMT to the matrix extract oxidizing glycine in the presence of a low initial concentration of H_4folate led to a linearity of the NADH production rate (Fig. 4) and to a decline of 5,10-CH_2-H_4folate attained at the equilibrium (Fig. 4). All these results together demonstrate that glycine decarboxylase and SHMT are not linked together and interact via a soluble pool of H_4folate.

The rate of glycine oxidation catalysed by the matrix extract exhibited saturation with increasing NAD^+ concentration, with an apparent K_m of 75 μM (Neuburger *et al.*, 1986). However, the product of the glycine

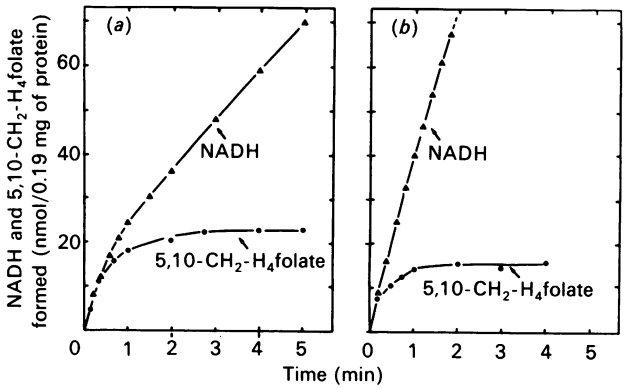

Fig. 4. Simultaneous measurement of NADH and 5,10-CH_2-H_4-folate formation during the course of glycine oxidation catalysed by a matrix extract isolated from pea leaf mitochondria.

The preparation of the matrix extract is described in the text. The standard reaction mixture contained 148 μM-H_4-folate, 0.19 mg of matrix extract protein (*a*) and 0.19 mg of matrix extract protein +35 μg of purified SHMT (*b*). Glycine oxidation was assayed at 25 °C by measuring the formation of either NADH at 340 nm, using a spectrophotometer, or 5,10-CH_2-H_4folate, using [2-^{3}H]glycine (1.1 TBq/mmol; Amersham International) and trapping the formaldehyde product isolated from 5,10-CH_2-H_4-folate as the dimedon complex (see the Materials and methods section).

oxidation, namely NADH, competitively inhibited the reaction when NAD^+ was the varied substrate at saturating concentrations of H_4folate and glycine (Neuburger *et al.*, 1986). Interestingly, the apparent K_i value (15 μM) for NADH was 5 times lower than the K_m for NAD^+. Since the reoxidation of the dihydrolipoyl moiety of the H-protein catalysed by lipoamide dehydrogenase utilized NAD^+ as the ultimate electron acceptor, we have examined pea leaf lipoamide dehydrogenase (L-protein) activity as a function of NAD^+ and NADH concentrations. Fig. 6 indicates that L-protein had, in contrast with the glycine decarboxylase/SHMT system (matrix extract), a relatively high K_m for NAD^+. On the other hand, the apparent K_i value for NADH was in the same range as the value reported for glycine decarboxylase/SHMT system. These results demonstrate, therefore, that the steady-state activity of the complex is sensitive to the NADH/NAD^+ molar ratios and strongly suggest that NAD^+ and NADH act directly on L-protein via a lipoyl moiety covalently bound to the H-protein, a constituent element of the glycine-cleavage system (Kikuchi & Hiraga, 1982). In support of this last suggestion, we have shown that neither the glycine–bicarbonate exchange reaction catalysed by P- and H-protein nor SHMT were inhibited by NADH (results not shown). Interestingly, since isolated lipoamide dehydrogenase had an apparent K_m for NAD^+ (Fig. 6) higher than the value reported for the matrix extract (Neuburger *et al.*, 1986), it is very likely that L-protein once associated with the glycine-cleavage system does not determine the final rate of glycine oxidation in the presence of saturating amounts of NAD^+. This view is strengthened by the fact that addition of purified L-protein to the enzymic extract in the presence of saturating amounts of NAD^+, glycine

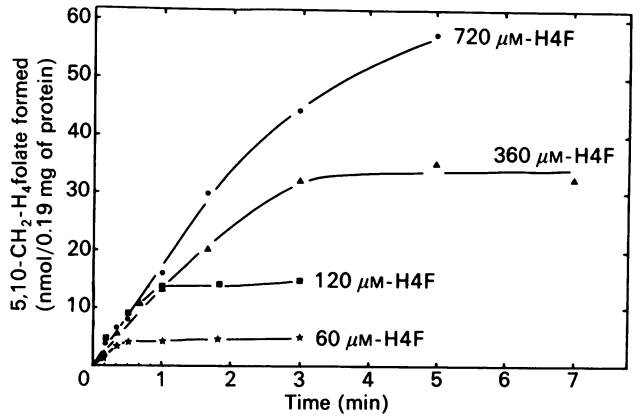

Fig. 5. Effect of H_4folate on 5,10-CH_2-H_4folate formation during the course of glycine oxidation catalysed by a matrix extract isolated from pea leaf mitochondria

The preparation of the matrix extract is described in the text. The standard reaction mixture contained 0.19 mg of matrix extract protein and various concentrations of H_4-folate (H4F) as indicated. 5,10-CH_2H_4folate formation was assayed at 25 °C using [2-^{3}H]glycine (1.1 TBq/mmol; Amersham International) and trapping the formaldehyde product isolated from 5,10-CH_2-H_4folate as the dimedon complex (see the Materials and methods section).

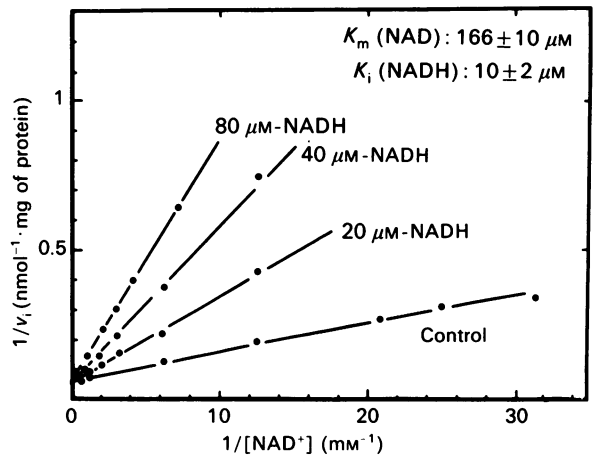

Fig. 6. Initial-velocity (v_i) patterns of dihydrolipoic acid oxidation by purified L-protein isolated from the glycine-cleavage system

The reaction media and the preparation of L-protein were described in the text. NAD^+ was the variable substrate and [NADH] was fixed at zero (control) or at various concentrations as indicated. Dihydrolipoic acid oxidations was assayed at 25 °C (see the text). The K_m for NAD^+ was 166±10 μM; the K_i for NADH was 10±2 μM.

and H_4folate did not change the rate of glycine oxidation (Table 4).

DISCUSSION

The experiments described concern the fragile enzyme system from leaf mitochondria, isolated as described by Neuburger *et al.* (1986) and capable of very rapidly oxidizing glycine to NADH and serine. It is obvious that the physiological integrity of this system is maintained at low ion concentrations and at a pH of about neutrality. Under these conditions, once released from the matrix space all the enzyme constituents of this 'complex', including H-protein (molecular mass 15.5 kDa) are retained on an XM-300 Diaflo membrane. On the other hand, all the protein components of the complex become separable non-associated polypeptides as the concentration of ions and pH of the medium are increased. Furthermore, since the glycine-cleavage system and SHMT were readily solubilized by freezing and thawing the mitochondria, the results imply that all the proteins of the glycine-cleavage system, as well as SHMT, are not firmly bound to the inner membrane, in contrast with previous suggestions (Sarojini & Oliver, 1983).

Two points of interest emerge from these studies with respect to the pyruvate dehydrogenase complex. Firstly there is a clear difference in the aggregation state of protein components of pyruvate dehydrogenase and glycine-cleavage complexes. It is apparent that, in contrast with pyruvate dehydrogenase, all the protein components of the glycine-cleavage system dissociate very easily and perhaps behave as separate components in the matrix space. This point requires further investigation. Interestingly, Walker & Oliver (1986*a*) have also shown that the glycine decarboxylase multienzyme complex from pea leaf mitochondria exists in solution predominantly as four separate enzymes after acetone extraction. Secondly, molecular-mass similarities between the purified lipoamide dehydrogenase of glycine decarboxylase (molecular mass 120 kDa) and pyruvate dehydrogenase (Reed, 1981) raise the question whether a unique protein is involved in the reoxidation of the dihydrolipoyl moieties of both lipoamide dehydrogenase-containing enzyme complexes or whether isoenzymes are involved. Preliminary results carried out by Walker & Oliver (1986*a*) indicate that a monoclonal antibody against the lipoamide dehydrogenase of pea leaf glycine cleavage complex produced indistinguishable inhibition profiles for both complexes. On the other hand, rat liver mitochondria contains two immunologically distinct forms of dihydrolipoamide dehydrogenase (Carothers *et al.*, 1987).

The results presented here strongly suggest that a soluble pool of H_4folate located in the matrix constitutes a biochemical link between the glycine-cleavage system and SHMT. It is very likely that the concentration of 5,10-CH_2-H_4folate attained at the equilibrium (that is, when its rate of formation by the glycine-cleavage-complex matches its rate of utilization by SHMT) is strongly dependent on the respective strengths of glycine cleavage and SHMT. In support of this suggestion we have shown that, when the activity of the glycine decarboxylase is powerful, 5,10-CH_2-H_4folate is maintained at a high level. Conversely, a large excess of SHMT decreased the level of 5,10-CH_2-H_4folate attained at the equilibrium. It was previously shown that glycine decarboxylase represents a large proportion of the matrix protein in green leaf mitochondria (Day *et al.*, 1985; Walker & Oliver, 1986*a*). Furthermore, we have shown that, after centrifugation on Percoll gradients, mitochondria from etiolated leaves were located in a band higher in the tube than those from mature leaves containing the glycine-cleavage/SHMT system, suggesting the mitochondria from etiolated leaves are lighter (Day *et al.*, 1985). If the large amount of glycine decarboxylase present in the matrix space is associated with a large pool of soluble H_4folate, we can speculate

that *in vivo* during the course of glycine oxidation a large pool of 5,10-CH_2-H_4folate is maintained in the matrix space owing to glycine decarboxylase activity continuously pushing SHMT towards serine production (SHMT catalysed a freely reversible reaction). Such a situation triggers a rapid rate of O_2 consumption. This is in contrast with the situation observed in mammalian mitochondria, where glycine added to intact mitochondria is unable to trigger O_2 consumption because glycine decarboxylase represents a minute fraction of the total matrix protein. In support of this suggestion, preliminary experiments carried out in this laboratory indicate that, during the greening process, the concentration of H_4folate in isolated purified mitochondria increased steadily. To date, long-chain polyglutamates (Coffin & Cossins, 1986; Cossins, 1987) have not been examined as substrates for glycine decarboxylase and SHMT, and the substrate specificity of these enzymes warrants further studies.

The data reported here also demonstrate that glycine-cleavage activity *in vivo* in green leaf mitochondria is regulated by the NADH/NAD^+ molar ratio and that NAD^+ and NADH act directly on L-protein via the lipoyl moiety covalently bound to the H-protein. The activity of the mitochondrial pyruvate dehydrogenase and α-oxo glutarate dehydrogenase complexes containing lipoamide dehydrogenase are also regulated by NADH, which is competitive with respect to NAD^+ (for a review, see Douce, 1985). However, in the case of pyruvate dehydrogenase an increase in the NADH/NAD^+ molar ratio decreases the steady-state activity of the complex by increasing the proportion of the phosphorylated inactive form of pyruvate dehydrogenase (Reed, 1981). Interestingly, the low K_m for NAD^+ of glycine oxidation by the enzymic extract, which is lower than those of other NAD^+-linked dehydrogenases (Douce, 1985), indicates that glycine cleavage competes favourably at the level of matrix NAD^+. In fact the preferential oxidation of glycine observed by several groups utilizing green leaf mitochondria is achieved by a dominance of complex I over both complex II and the external NADH dehydrogenase of the respiratory chain (for a review, see Douce, 1985), by the ability of glycine decarboxylase to compete favourably at the level of NAD^+ (present results) and by the high concentration of glycine decarboxylase present in the matrix space. However, it is very likely that green cells *in vivo* will generally be respiring in States between 4 and 3, especially under high-light conditions, because the cytosolic ATP/ADP ratio is considerable. That is, the rate at which a green cell respires is limited by the availability of ADP for oxidative phosphorylation. Such a situation will lead to an increase in the matrix NADH/NAD^+ ratio impeding glycine-cleavage functioning. Since NADH strongly inhibits glycine decarboxylase, we are therefore forced to imagine that, *in vivo*, NADH produced during the course of glycine oxidation is reoxidized very rapidly by oxaloacetate, owing to the malate dehydrogenase located in the matrix space working in the reverse direction. In support of this suggestion a very powerful oxaloacetate carrier has been characterized in all the plant mitochondria isolated so far (Douce & Bonner, 1972; Woo & Osmond, 1976) with a very high affinity to its substrate. This rapid malate–oxaloacetate transport shuttle appears to play an important role in the photorespiratory cycle in catalysing the transfer of reducing equivalents generated in the mitochondria during glycine oxidation to the peroxisomal compartment for the reduction of β-hydroxypyruvate (Ebbighausen *et al.*, 1985).

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