Isolation of a large complex from the matrix of pea leaf mitochondria involved in the rapid transformation of glycine into serine

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The glycine cleavage system associated with serine hydroxymethyltransferase has been extracted as a fully active complex from pea leaf mitochondria. Some biochemical properties of this complex were studied.

Mitochondria *Photorespiration* *Glycine oxidation* *Serine hydroxymethyltransferase* *Glycine cleavage* *Tetrahydrofolate*

1. INTRODUCTION

Glycine and serine are intermediates in the photorespiratory metabolism of glycolate to 3-phosphoglycerate. 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, serine and NADH [1,2]. No glycine oxidation activity is present in mitochondria from non-green or etiolated tissues [3,4]. Glycine is oxidized in the matrix space by the glycine cleavage system (aminomethyltransferase EC 2.1.2.10) to CO_2, NH_3 and 5,10-methylenetetrahydrofolate [5,6]. The latter compound produced reacts with a second mole of glycine to form serine in a reaction catalyzed by serine hydroxymethyltransferase (EC 2.1.2.1) [7]. According to several authors (review [8]) both the decarboxylase and bicarbonate exchange activities are lost when the inner membrane is ruptured. We report here for the first time the isolation from pea leaf mitochondria of a large multienzyme complex containing the glycine cleavage system (glycine decarboxylase) associated with serine hydroxymethyltransferase (SHMT) and capable to oxidize very rapidly glycine to yield serine and NADH. In addition in this paper we describe the biochemical properties of this complex.

2. MATERIALS AND METHODS

2.1. *Materials*

Pea (*Pisum sativum* L. Var Douce Provence) plants were grown from seeds in vermiculite for 15 days under a 12 h photoperiod of warm white light from fluorescent tubes (10–40 $\mu E/m^2$ per s of photosynthetically active radiation) at 22°C. The plants were grown at high density (20–30 plants per 100 cm^2). The plants were watered every day with one-half strength Hoagland's nutrient solution. Approx. 1 kg of fully expanded leaves was used.

2.2. *Isolation of mitochondria*

Mitochondria were isolated and purified from pea leaves as described [4], using self-generating Percoll gradients and a linear gradient of 0–10% (w/v) PVP-25 (top of bottom). The mitochondria were found in a tight, white band near the bottom of the tube while 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

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mM EDTA and 1 mM β-mercaptoethanol at approx. 100 mg protein/ml. The yield of mitochondria for 1 kg of pea leaves was approx. 100 mg protein.

O_2 uptake was measured at 25°C with a Clark-type O_2 electrode purchased from Hansatech (Norfolk, England). 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 volume of 1 ml. The O_2 concentration in air-saturated medium was assumed to be 250 μM. With 10 mM glycine as substrate, the respiratory control index was routinely better than 4 and the state III rate of O_2 uptake was high than 100 nmol/min per mg of protein. The mitochondria were better than 95% intact as judged by their impermeability to cytochrome *c* [9].

2.3. *Solubilization of the glycine cleavage/serine hydroxymethyltransferase complex*

Pea leaf mitochondria (100 mg protein) were diluted in 100 ml of lysis buffer containing 5 mM Mops, 5 mM Tris, 1 mM β-mercaptoethanol, 20 μM pyridoxal-P, 1 mM serine, 1 mM octyl-β-D-glucopyranoside (Calbiochem), pH 7.0. Total release of the matrix protein was achieved by 3 cycles of freezing and thawing. The mitochondrial suspension was frozen by placing it at N_2 liquid temperature for 2 min. The frozen mitochondrial suspension was then placed 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 rpm in a Beckman SW-40 rotor) to remove all of the mitochondrial membranes. The high molecular mass complexes (above 300000 Da) from the matrix space 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 (enzymatic extract, final volume 2 ml) insofar as the pH is maintained around neutrality and the medium exhibits a low ionic strength.

2.4. *Measurement 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 tetrahydrofolate. The standard reaction mixture contained in a volume of 400 μl, 5 mM Mops, 5 mM Tris (pH 7.2), 1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 1 mM EGTA, 20 μM pyridoxal phosphate, 350 μM tetrahydrofolate, 5 μg antimycin A and known quantities of enzymatic extract. The medium was bubbled with argon (in the presence of atmospheric O_2 there was a rapid oxidation of tetrahydrofolate to dihydrofolate which has an absorption maximum at 282 nm). 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 cold 1 N 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 analyzed in sodium citrate buffers with an amino acid analyzer (model 118 C, Beckman Instrument, Palo Alto, CA).

3. RESULTS

High molecular mass proteins from the mitochondrial matrix retained on an XM-300 Diaflo membrane produced an enzymatic extract exhibiting high rates of glycine oxidation in the presence of tetrahydrofolate and pyridoxal-P (fig.1). During the course of glycine oxidation NAD^+ and serine were formed equally indicating that the glycine cleavage system and SHMT are present in the enzymatic extract and operate in a concerted manner (fig.2). Carboxymethoxylamine [10] strongly inhibited glycine oxidation by the enzymatic extract (fig.1). The glycine saturation curve was hyperbolic with a K_m (glycine) value of 6.5 mM (not shown). We have observed that a linear relationship between NADH formation rates and the amount of enzyme occurred only at relatively high concentrations of the enzymatic extracts (fig.3) (see also [6], in the case of glycine decarboxylase solubilized from pea leaf mitochondria as an acetone powder).

Fig.4 indicates the effect of tetrahydrofolate (THF) concentration on glycine oxidation by the enzymatic extract. At low THF concentrations the

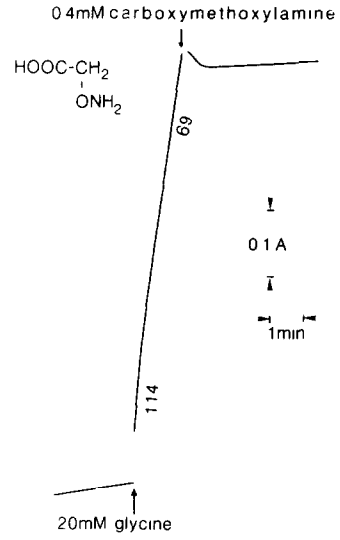

Fig.1. Glycine oxidation catalyzed by an enzymatic extract isolated from pea leaf mitochondria. The preparation of the enzymatic extract is described in the text. The reaction medium (see section 2) contained 0.3 mg enzymatic extract protein. Glycine oxidation was assayed at 25°C by measuring the formation of NADH at 340 nm using a Kontron (Uvikon-810) spectrophotometer. The values along the trace refer to nmol of NADH formed/min per mg of protein.

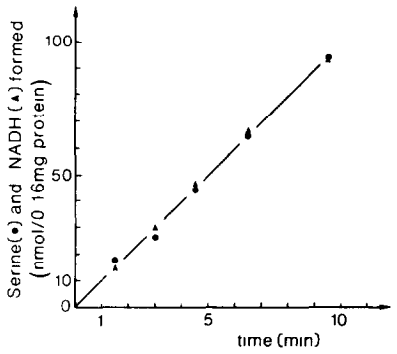

Fig.2. Time course serine and NADH accumulation during glycine oxidation by an enzymatic extract isolated from pea leaf mitochondria. The preparation of the enzymatic extract is described in the text. The reaction medium (see section 2) contained 1.3 mg enzymatic extract protein. Final pH was 7.2 and final volume of the reaction medium was 400 μl. The reaction was initiated by addition of 5 mM glycine. Serine formation was determined from 50 μl-aliquots (0.16 mg protein) as described in section 2.

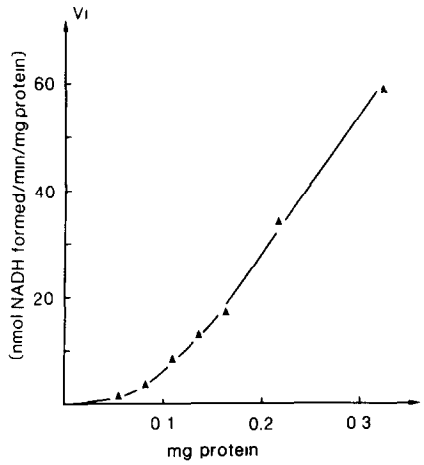

Fig.3. The effect of increasing amounts of proteins on the rate of glycine oxidation by an enzymatic extract isolated from pea leaf mitochondria. The reaction medium and the preparation of the enzymatic extract are described in the text. Glycine oxidation was assayed at 25°C by measuring the formation of NADH at 340 nm using a Kontron (Uvikon-810) spectrophotometer.

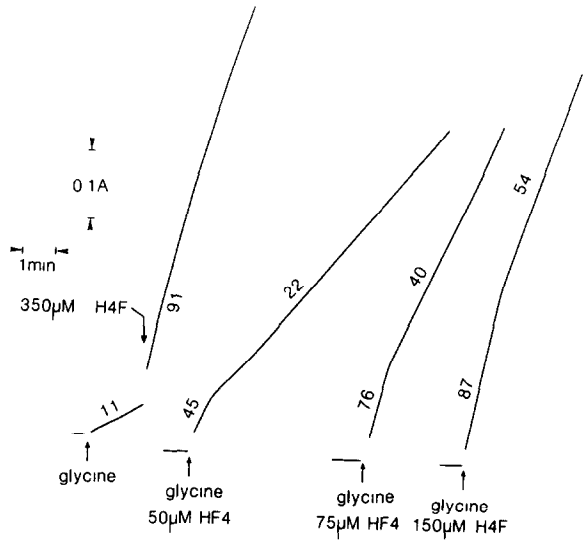

Fig.4. The effect of tetrahydrofolate on glycine oxidation catalyzed by an enzymatic extract isolated from pea leaf mitochondria. The preparation of the enzymatic extract is described in the text. The reaction medium (see section 2) contained 0.3 mg enzymatic extract protein. Glycine oxidation was assayed at 25°C by measuring the formation of NADH at 350 nm using a Kontron (Uvikon-810) spectrophotometer. The values along the traces refer to nmol of NADH formed/min per mg of protein.

rate of glycine oxidation was clearly biphasic. This would suggest that the rate-limiting step of glycine oxidation may be the regeneration of THF from methylene-THF by SHMT. In the absence of pyridoxal-P glycine oxidation rate decreased rapidly until the reaction stopped completely (fig.5). Addition of pyridoxal-P triggered, after a short lag phase, the full rate of glycine oxidation. The apparent K_m for pyridoxal-P was estimated to be 2 μM (not shown). Such a result suggests that pyridoxal-P, which is required for the full activity of both complexes, is easily released from SHMT during the course of the preparation of the enzymatic extract. The rate of glycine oxidation also exhibited saturation with increasing NAD^+ concentration with an apparent K_m of 75 μM (fig.6).

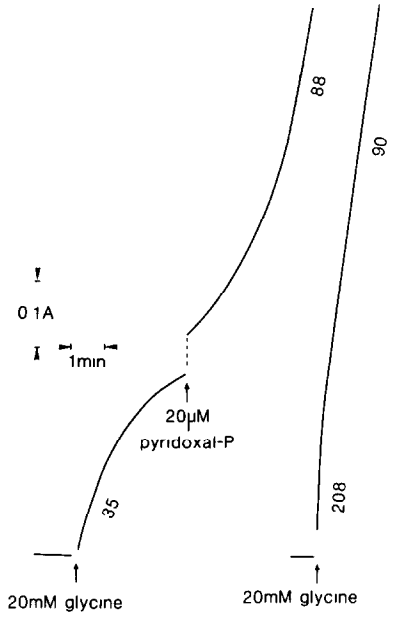

Fig.5. The effect of pyridoxal-P on glycine oxidation catalyzed by an enzymatic extract isolated from pea leaf mitochondria. The preparation of the enzymatic extract is described in the text. The reaction medium (see section 2) contained 0.3 mg enzymatic extract protein. Glycine oxidation was assayed at 25°C by measuring the formation of NADH at 340 nm using a Kontron (Uvikon-810) spectrophotometer. The values along the traces refer to nmol of NADH formed/min per mg of protein. Left trace: no pyridoxal-P in reaction medium, 20 μM added as indicated. Right trace: 20 μM pyrixodal-P included in the reaction medium.

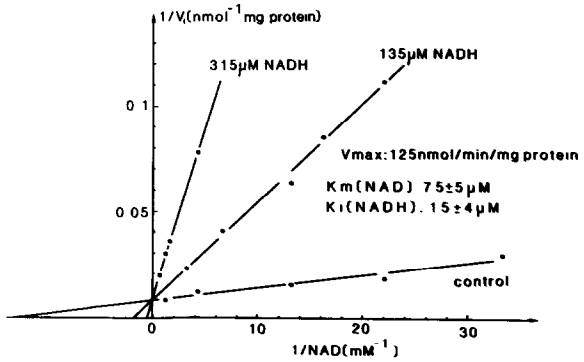

Fig.6. Initial velocity patterns of glycine oxidation by an enzymatic extract isolated from pea leaf mitochondria. The reaction medium and the preparation of the enzymatic extract are described in the text. NAD^+ was the variable substrate and NADH fixed at 0 (control), 135 and 315 μM. Glycine oxidation was assayed at 25°C by measuring the formation of NADH at 340 nm using a Kontron (Uvikon-810) spectrophotometer.

The product of the glycine oxidation, NADH, competitively inhibited the reaction when NAD^+ was the varied substrate at saturating concentrations of THF and glycine (fig.6). Interestingly, the apparent K_i value for NADH was 5-times lower than the K_m for NAD^+. These results strongly suggest that in vivo glycine decarboxylase activity in green leaf mitochondria is regulated by the NADH-NAD^+ molar ratio. This enzymatic extract exhibited no conspicuous pH dependence between 6.5 and 7.5 (not shown).

Increasing the ionic strength in the medium by adding 100 mM KCl (fig.7) or $(NH_4)_2SO_4$ (not shown) prevented glycine oxidation. However salt removal by ultrafiltration using an Amicon YM-5 membrane restored the full activity. Likewise at a constant ionic strength the complex formation appeared to be favoured at relatively low pH (not shown). These results demonstrate that high ionic strength and pH disrupt in a reversible way the fairly stable complex involved in glycine oxidation. In fact preliminary experiments carried out in our laboratory have clearly shown that the affinity of P-protein to bind H-protein, two proteins directly involved in the bicarbonate exchange catalyzed by the glycine decarboxylase complex [5,6], decreases as the concentration of ions and pH in the medium increase. In other words H-protein is easily re-

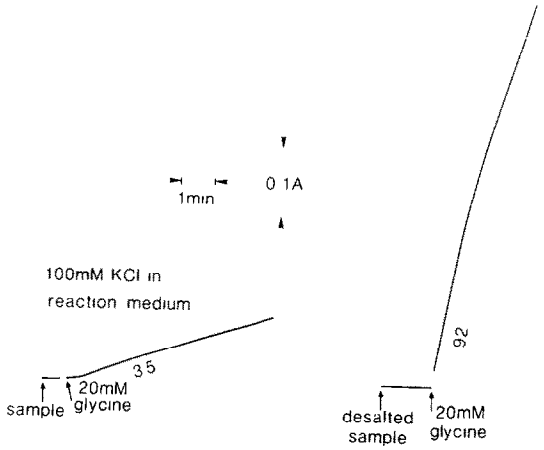

Fig.7. The effect of high ionic strength on glycine oxidation catalyzed by an enzymatic extract isolated from pea leaf mitochondria. The preparation of the enzymatic extract is described in the text. The reaction medium (see section 2) contained 0.3 mg enzymatic extract protein. Glycine oxidation was assayed at 25°C by measuring the formation of NADH at 340 nm using a Kontron (Uvikon-810) spectrophotometer. The values along the traces refer to nmol of NADH formed/min per mg of protein. Left trace: 100 mM KCl included in the reaction medium. Right trace: the desalted sample was obtained by filtration of the reaction medium containing 100 mM KCl on an Amicon YM5 membrane.

leased from the glycine decarboxylase complex at high ionic strength and pH.

4. DISCUSSION

These results demonstrate for the first time the isolation from green leaf mitochondria of an active complex capable of oxidizing very rapidly glycine to NADH and serine. It is obvious that the physiological integrity of this complex is maintained if three criteria are met: (i) the preparation of the enzymatic extract must be carried out at extremely low ion concentrations and at pH around neutrality in order to prevent the release of the H protein from the glycine decarboxylase complex; (ii) the suspending medium must contain saturating amounts of critical cofactors such as THF and pyridoxal-P; (iii) glycine oxidation must be carried out under strict anaerobic conditions.

The data reported here raise the problem of the binding of THF to glycine decarboxylase and SHMT and suggest that most of the THF is not firmly bound to this multi-enzymatic complex. It is very likely that in vivo glycine decarboxylase and SHMT are 'linked together' via a soluble pool of THF, which does not react with O_2.

The data reported here also demonstrate that in vivo glycine decarboxylase activity in green leaf mitochondria is regulated by NADH-NAD^+ molar ratios. On the other hand the low K_m for NAD^+ of glycine oxidation which is lower than those of other NAD^+-linked dehydrogenases (review [8]) indicates that glycine decarboxylase competes favourably at the level of matrix NAD^+. In fact the preferential oxidation of glycine observed by several groups [11–13] is achieved by a dominance of complex I over both complex II and the external NADH dehydrogenase of the respiratory chain (review [8]), by the ability of glycine decarboxylase to compete favourably at the level of NAD^+ (this result) and by the huge concentration of glycine decarboxylase present in the matrix space [4,6].

REFERENCES

[1] Keys, A.J. (1980) in: The Biochemistry of Plants, vol.5, Amino Acids and Derivatives (Miflin, B.J. ed.) pp.359–374, Academic Press, New York.
[2] Tolbert, N.E. (1980) in: The Biochemistry of Plants, vol.2, Metabolism and Respiration (Davies, D.D. ed.) pp.487–523, Academic Press, New York.
[3] Gardeström, P., Bergman, A. and Ericson, I. (1980) Plant Physiol. 65, 389–391.
[4] Day, D.A., Neuburger, M. and Douce, R. (1985) Aust. J. Plant Physiol. 12, 119–130.
[5] Kikuchi, G. and Hiraga, K. (1982) Mol. Cell. Biochem. 45, 137–149.
[6] Walker, J.L. and Oliver, D.J. (1986) J. Biol. Chem. 261, 2214–2221.
[7] Schirch, L. and Peterson, D. (1980) J. Biol. Chem. 255, 7801–7806.
[8] Douce, R. (1985) Mitochondria in Higher Plants: Structure, Function and Biogenesis, Academic Press, New York.
[9] Douce, R., Christensen, E.L. and Bonner, W.D. (1972) Biochim. Biophys. Acta 275, 148–160.
[10] Sarojini, G. and Oliver, D.J. (1985) Plant Physiol. 77, 786–789.
[11] Bergman, A. and Ericson, I. (1983) Physiol. Plant 59, 421–427.
[12] Dry, I.B., Day, D.A. and Wiskich, J.T. (1983) FEBS Lett. 158, 154–158.
[13] Day, D.A., Neuburger, M. and Douce, R. (1985) Aust. J. Plant Physiol. 12, 219–228.