Stoichiometry of Carbon Dioxide Release and Oxygen Uptake During Glycine Oxidation in Mitochondria Isolated from Spinach (*Spinacia oleracea*) Leaves

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Mitochondria isolated from spinach (*Spinacia oleracea*) leaves oxidized glycine with a stoichiometry of CO₂ evolution to O₂ uptake of 2:1. In the absence of added substrate, the mitochondria exhibited an extremely low endogenous rate of O₂ uptake.

It has been established that glycine decarboxylase activity in plants is localized in the mitochondria of photosynthetic tissue (Kisaki *et al.*, 1971*a,b*; Bird *et al.*, 1972*a,b*; Collins *et al.*, 1975; Woo & Osmond, 1976; Moore *et al.*, 1977; Rathnam, 1979). Kisaki *et al.* (1971*a,b*) reported that glycine decarboxylation by spinach leaf mitochondrial fractions was faster in N₂ than in air and required NAD⁺, pyridoxal phosphate and tetrahydrofolate as cofactors. Bird *et al.* (1972*a,b*), who used tobacco (*Nicotiana tabacum*) leaf mitochondria, and Collins *et al.* (1975), who used *Euglena* mitochondria, reported that the reaction required aerobic conditions, was linked to the electron-transport chain, and coupled to the synthesis of two (Bird *et al.*, 1972*a*) or one (Collins *et al.*, 1975) molecule of ATP for each molecule of serine formed. Woo & Osmond (1976) suggested that spinach leaf mitochondria contain at least two glycine-decarboxylating systems, one stimulated by ADP and coupled to the electron-transport chain and the other stimulated by NAD⁺ and oxaloacetate, but not coupled directly to electron transport.

More recent work (Douce *et al.*, 1977; Moore *et al.*, 1977; Rathnam, 1979) has confirmed that glycine oxidation is linked to electron transport and is coupled to three phosphorylation sites (and not one or two as indicated previously; Bird *et al.*, 1972*a*; Collins *et al.*, 1975). Moore *et al.* (1977) reported rates of glycine decarboxylation in spinach leaf mitochondria that were similar to the rate of glycine-dependent O₂ uptake. For example, in State 3 (in the presence of ADP), the rates of O₂ consumption and CO₂ evolution with glycine as substrate were the same (96 nmol/min per mg of protein and 97.2 nmol/min per mg of protein respectively; each value was the mean for four experiments). Rathnam (1979) reported a similar stoichiometry with mesophyll mitochondria from a C₃ grass (*Panicum bisulcatum*)

(12.5 μmol of CO₂ evolved/h per mg of chlorophyll and 12.8 μmol of O₂ consumed/h per mg of chlorophyll); decarboxylation was measured in State 2 and O₂ uptake in State 4. However, previous work by Bird *et al.* (1972*b*) indicated that tobacco leaf mitochondria catalysed the conversion of two molecules of glycine into one molecule of serine, with approximately one atom of O₂ taken up for each molecule of serine and CO₂ formed.

We have determined the stoichiometry of CO₂ formation to O₂ uptake in spinach leaf mitochondria supplied with glycine as substrate by measuring both parameters in the same experiment and not in two separate experiments, as in previous studies (see e.g. Bird *et al.*, 1972*b*; Moore *et al.*, 1977; Rathnam, 1979).

Materials and Methods

Reagents were of the highest purity available from Sigma, St. Louis, MO, U.S.A. [1-¹⁴C]Glycine and NaH¹⁴CO₃ were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Local field-grown spinach (*Spinacia oleracea* L.) leaves were obtained and used immediately. Mitochondria were isolated from spinach leaves by using a modification of the method of Douce *et al.* (1977). De-ribbed leaf material was homogenized for 3–4 s with a Polytron homogenizer (PT 35K probe; setting 7) in a chilled grinding medium containing 0.3 M-mannitol, 4 mM-cysteine, 1 mM-EGTA, 30 mM-Mops, pH 7.5, 0.2% bovine serum albumin and 0.6% insoluble polyvinylpyrrolidone (approx. 0.4 kg of leaf tissue to 1.5 litres of medium). The homogenate was squeezed through four layers of cheesecloth and centrifuged at 1200g for 4 min to remove chloroplasts and cell debris. The supernatant was then centrifuged at 10000g for 10 min. The pellets were resuspended in approx. 40 ml of wash medium containing 0.4 M-mannitol, 25 mM-Tes, pH 7.6, and 0.1% bovine serum albumin with a Teflon/glass homogenizer and centrifuged at 10000g for 10 min. The pellet was

Abbreviations used: Mops, 4-morpholinepropane-sulphonic acid; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid.

resuspended in approx. 4 ml of wash medium. Typical mitochondrial preparations oxidized glycine with respiratory control ratios of between 2.4–3.3.

Mitochondrial respiration was measured polarographically by using a Rank oxygen electrode (Rank, Bottisham, Cambridge, U.K.) at 25°C in a standard reaction medium [modified from Laties (1973)] containing 0.4 M-mannitol, 50 mM-Tes, pH 7.2, 2 mM-MgSO₄, 5 mM-KH₂PO₄ and 0.1% bovine serum albumin. The O₂ concentration in air-saturated medium was taken as 240 μM (Estabrook, 1967).

Glycine decarboxylation was measured as ¹⁴CO₂ released from [1-¹⁴C]glycine. Three or four 50 μl samples were taken directly from the reaction vessel of the Rank electrode over a time course of 0–7 min. The samples were injected into a serum-capped vial containing 400 μl of 0.5 M-H₂SO₄. During a 30–60 min incubation, the ¹⁴CO₂ released was trapped in 200 μl of methylbenzethonium hydroxide contained in a plastic cup suspended inside the sealed vial. The absorbed ¹⁴CO₂ was determined by liquid-scintillation spectroscopy. A known amount of NaH¹⁴CO₃ in reaction medium was sampled from the reaction vessel in an identical manner. Recovery was 96–100%

and was unchanged if left in the reaction vessel for 6 min.

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard, and chlorophyll determined in ethanol by the method of Wintemans & De Mots (1965). The amount of mitochondrial protein was corrected for the contribution of broken thylakoids by assuming a protein-to-chlorophyll ratio of 7 in broken thylakoids (Lilley *et al.*, 1975).

Results and Discussion

Fig. 1 shows the simultaneous evolution of CO₂ and uptake of O₂ by spinach leaf mitochondria supplied with [1-¹⁴C]glycine as substrate (note that the scale for ¹⁴CO₂ released is double that for O₂ uptake). The direct relationship between glycine decarboxylation and glycine-dependent O₂ uptake is apparent, since both processes are stimulated to a similar extent by ADP and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and are both inhibited by the electron-transport inhibitor antimycin A. Isoniazid (pyridine-4-carboxylic acid hydrazide), a

Fig. 1. Glycine decarboxylation and glycine-dependent O₂ uptake in spinach leaf mitochondria

The reaction mixture contained 1.0 ml of mitochondrial assay medium, 0.05 ml of mitochondrial extract and 10 mM-glycine containing 2.5 μCi of [1-¹⁴C]glycine. Additions were as indicated: (a) 2.0 μmol of ADP (added before substrate); (b) none; (c) 2.0 nmol of FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone); (d) 20 μmol of isoniazid; (e) 3.5 nmol of antimycin A; (f) 2.0 μmol of NAD⁺; (g) 2.0 μmol of oxaloacetate. O₂ uptake and CO₂ release were measured as described in the Materials and Methods section.

Table 1. *Glycine decarboxylation in spinach leaf mitochondria*

Values measured are means for two experiments. The reaction mixture is as described in Fig. 1. O₂ uptake and CO₂ release were measured simultaneously as described in the Materials and Methods section. The numbers in parentheses refer to percentage of control value. In the absence of added glycine, the endogenous rate of O₂ uptake was 2.1 nmol/min per mg of protein. Abbreviation used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Modification to reaction mixture	O ₂ uptake (nmol/min per mg of protein)		CO ₂ release (nmol/min per mg of protein)		CO ₂ /O ₂
None (State 2)	14.9	(100)	30.0	(100)	2.01
+2.0mM-ADP	27.0	(181)	51.8	(173)	1.92
+20mM-Isoniazid	7.1	(48)	14.2	(47)	2.00
+3.5 μ M-Antimycin A	5.7	(38)	10.8	(36)	1.89
+2.0 μ M-FCCP	25.0	(168)	50.2	(167)	2.01
+2.0mM-Oxaloacetate	4.5	(30)	67.3	(224)	14.90
+2.0mM-NAD ⁺	11.9	(80)	16.1	(54)	1.35

specific inhibitor of serine synthesis from glycine *in vivo* (Pritchard *et al.*, 1962) inhibited both glycine decarboxylation and glycine-dependent O₂ uptake to a similar extent. Addition of oxaloacetate stimulated glycine decarboxylation more than 2-fold and had a transient inhibitory effect on glycine-dependent O₂ uptake. It is proposed that the NADH produced by glycine decarboxylation would be available to reoxidize oxaloacetate in the presence of mitochondrial malate dehydrogenase, rather than being oxidized by the electron-transport chain. Except as regards the stoichiometry of the reaction, the results described above confirm the previous reports of Moore *et al.* (1977) and Rathnam (1979).

Table 1 presents the stoichiometry between CO₂ release and O₂ uptake by spinach leaf mitochondria with glycine as substrate under a variety of conditions. It should be noted that, in the absence of added substrate, the endogenous rate of O₂ uptake by spinach leaf mitochondria was extremely low. The stoichiometry between CO₂ release and O₂ uptake is approx. 2 under most conditions (e.g., State 2, State 3, +carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, +antimycin A, +isoniazid). This stoichiometry is illustrated in Fig. 1, since the time courses for ¹⁴CO₂ released and O₂ uptake are nearly superimposed in most cases, with the scale of the former double that of the latter. When oxaloacetate was added, the ratio of CO₂ released to O₂ consumed increased greatly, as described above. Addition of NAD⁺ resulted in a small inhibition of glycine-dependent O₂ uptake and a 50% inhibition of glycine decarboxylation (see Fig. 1 and Table 1). Previous workers reported either no effect of NAD⁺ on glycine decarboxylation and glycine-dependent O₂ uptake (Moore *et al.*, 1977; Rathnam, 1979) or a large stimulation of decarboxylation (Woo & Osmond, 1976). We can offer no ready explanation for the inhibitory effect of NAD⁺ in our experiments.

The stoichiometry of approx. 2 (CO₂ released/O₂ consumed; Fig. 1 and Table 1) is in close agreement with both the theoretical stoichiometry of the overall reaction (Schnarrenberger & Fock, 1976) and previous results with tobacco mitochondria (Bird *et al.*, 1972b). The recent results of Moore *et al.* (1977) and Rathnam (1979) suggest a stoichiometry of 1, and this may be due to the inaccuracy of assaying decarboxylation and O₂ uptake separately, rather than simultaneously as described in the present paper.

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