# INTRAMITOCHONDRIAL LOCALISATION OF GLYCINE DECARBOXYLASE IN SPINACH LEAVES

A.L. Moore, C. Jackson, B. Halliwell, J.E. Dench and D.O. Hall

University of London King's College, Department of Plant Sciences, 68 Half Moon Lane, London SE24 9JF. U.K.

Received August 1, 1977

Summary: Intact spinach leaf mitochondria are capable of oxidising glycine with good respiratory control and the oxidation is coupled to 3 phosphorylation sites. The intramitochondrial localisation of glycine decarboxylation has been studied and it is demonstrated that the enzyme system is associated with the inner membrane of spinach leaf mitochondria. Both glycine decarboxylation and glycine dependent $O_2$ uptake are stimulated by ADP and FCCP and are sensitive to electron transport inhibitors. Both processes showed no requirements for co-factors. We suggest that glycine decarboxylase is coupled to the electron transport chain via an $NAD^+$-linked system and that during rapid photorespiration glycine oxidation synthesises considerable amounts of ATP outside of the chloroplast.

Introduction: It is well documented that mitochondria from the leaves of higher plants can catalyse the decarboxylation of glycine, coupled to the formation of serine [1,2]. This decarboxylation reaction is currently considered to be the major source of the $CO_2$ released during photorespiration [1,2]. Since a large amount of the $CO_2$ fixed by illuminated leaves is released again by photorespiration [3], mitochondrial glycine decarboxylation is a major metabolic pathway in the leaf.

Kisaki et al [4] found that glycine decarboxylation by spinach leaf mitochondrial fractions was faster in $N_2$ than in air and required $NAD^+$, pyridoxal phosphate and tetrahydrofolate as cofactors. However, glycine decarboxylation by tobacco [5,6] or Euglena [7] mitochondrial fractions was not affected by addition of these cofactors and appeared to be linked to the electron-transport chain, being coupled to the synthesis of 2 [5,6] or 1 [7] ATP molecule for each serine molecule formed. However, the mitochondrial fractions used in these experiments showed very low rates of glycine decarboxylation and no respiratory control was evident.

Woo and Osmond [8] suggested that spinach leaf mitochondria contain at least 2 glycine decarboxylating systems. One system was suggested to be





ISSN 0006-291X

directly coupled to electron transport but the other, which was stimulated by addition of oxaloacetate and $NAD^+$, was not. Again, the mitochondrial fractions used showed poor respiratory control and low P/O ratios.

A technique for isolating mitochondria of high integrity from spinach leaves has recently been devised [9]. These mitochondria oxidise intermediates of the Krebs cycle with good respiratory control and ADP/O ratios comparable to other mitochondria from plant tissues.

Although the system catalysing the cleavage of glycine has been considered to be located in the mitochondrion [4-8], a detailed study of the properties of glycine decarboxylation, its linkage to the electron transport chain and its intramitochondrial localisation in intact spinach leaf mitochondria has not been reported. We have found only one glycine decarboxylating system which is located in the inner membrane and is directly linked to the electron transport chain presumably via an internally bound NADH dehydrogenase.

Materials and Methods: Reagents were of the highest purity available from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K., or from BDH Chemicals Ltd., Poole, Dorset, U.K. [1-$^{14}$C] glycine was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. Rotenone, antimycin A, and tetrahydrofolic acid were obtained from Sigma Chemical Co. Kingston-upon-Thames, PHMS from Aldrich Chemical Co. FCCP from Boehringer Mannheim (London).

Fresh spinach (Spinacia oleracea L.) leaves were obtained from New Covent Garden and used immediately. Mitochondria from spinach leaves were isolated according to the method of Douce et al. [9]. In this technique young spinach leaves (approximately 0.5 kg in 2ℓ of grinding medium) were homogenised for 2 seconds at low speed in a grinding medium containing 0.3M mannitol, 4mM cysteine, 1mM EDTA, 30mM MOPS buffer pH 7.5, 0.2% BSA and 0.6% insoluble polyvinylpyrollidine in a 1 gallon Waring blender. The mitochondria were then separated from broken cell material, chloroplasts and nuclei by filtration through 6 layers of cheesecloth followed by two cycles of differential centrifugation.

Mitochondrial respiration was measured polarographically using a Hansatech oxygen electrode (King's Lynn, U.K.) in a 2.5 ml closed cell at 25°. Oxygen consumption measurements were performed in a reaction medium (2.3 ml) containing 0.3M Mannitol, 5mM $MgCl_2$, 10mM KCl, 10mM phosphate buffer pH 7.2 and 0.1% BSA (Medium A). The oxygen concentration in air-saturated medium was taken as 250 µM [10].

Abbreviations: Butyl-PBD, 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole; PHMS, 2-pyridylhydroxymethane sulphonic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MOPS, 3-(N-Morpholino)-propane sulphonic acid; BSA, bovine serum albumin.

The decarboxylase activity of the mitochondria was measured as the release of $^{14}CO_2$ from $^{14}C$-labelled substrates. The reaction mixtures containing 2.0 ml of Medium A and 0.05 ml of mitochondrial extract were incubated with shaking at 25° in tightly stoppered 20 ml flasks. The reaction was started by the addition of 5 mM glycine containing 0.15 µCi [1-$^{14}C$] glycine. Each flask was fitted with a small plastic tube containing 0.2 ml of 20% (w/v) KOH to collect the $^{14}CO_2$. 0.2 ml of 2.5M- $H_2SO_4$ was added to the reaction mixture after the appropriate time and incubation at 25° was continued for a further 60 min before sampling the KOH. The small tube was removed and 0.1ml of its contents added to 4.0 ml scintillation fluid (composition: 7g of butyl-PBD and 80g of napththalene dissolved in 600 ml toluene and 400 ml of 2-methoxyethanol). Samples were counted for radioactivity in a Packard liquid scintillation counter. All assays were accompanied by controls in which mitochondria were omitted, or by using mitochondria that had been heated at 100°C for 10 min. The rates of non-enzymatic decarboxylation measured were less than 10% of the enzymic rates, and the results presented have been corrected for these non-enzymatic rates. The net rate of $^{14}CO_2$ release from all of the substrates was proportional to the amount of extract added.

Total protein was determined by the Folin-Ciocalteau phenol reagent [11]. Chlorophyll was extracted from the mitochondrial pellet in 80% acetone and measured according to Arnon [12]. On an assumption of a protein to chlorophyll ratio of 7 in broken thylakoids [13], the amount of mitochondrial protein can be corrected for the contribution of the broken thylakoids. Crystalline BSA was used as a standard.

Results: Fig. 1a indicates that spinach leaf mitochondria are capable of oxidising glycine as a substrate with good respiratory control ($3.2 \pm 0.3$) and that its oxidation is coupled to three phosphorylation sites [9]. This result suggests that glycine oxidation is linked to the electron transport chain via an endogenous $NAD^+$-linked system similar to other respiration-linked dehydrogenases [14]. Fig. 1b presents further evidence confirming this idea. In the presence of 20 µM rotenone, a Site I specific electron transport inhibitor, glycine oxidation (State 3) is inhibited by 56% and coupled to only two phosphorylation sites. Similar results have been observed with malate as substrate. Residual oxidation was strongly inhibited by 1 µg/ml antimycin A or 1mM KCN.

Fig. 2 shows glycine decarboxylation by mitochondria measured by $^{14}CO_2$ release. It may be seen from Fig. 2 that following a lag period of 1-2 min. $^{14}CO_2$ release proceeds linearly for only about 10 min. after which the rate decreases substantially. In the rat liver system it has been reported that decarboxylation is linear for more than 60 minutes, however rates are substantially lower than the ones reported in this communication (10 nmoles.

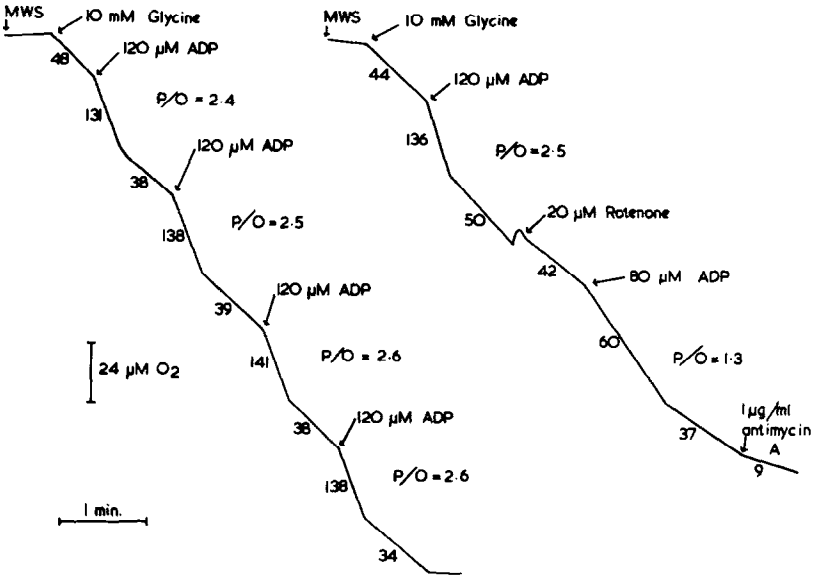


Figure 1

Oxidation of glycine by spinach leaf mitochondria

The incubation medium (2.4 ml) contained 0.3M Mannitol, 10mM KCl, 5mM $MgCl_2$, 10mM phosphate buffer pH 7.2, 0.1% BSA and approximately 1.6 mg mitochondrial protein. Rates of oxygen uptake are in nmol.min.$^{-1}$ mg protein$^{-1}$. The concentrations given are the final concentration in the reaction medium.

10 min$^{-1}$.mg protein$^{-1}$. cf.15) and the decarboxylation is not linked to the electron transport chain [16]. The decrease in $^{14}CO_2$ release could be due to mitochondrial breakage, since osmotic breakage or treatment with detergents caused irreversible loss of activity. It is also well known that isolated plant mitochondria rapidly deteriorate in dilute suspensions during long incubation periods [17,18]. Consequently mitochondria used for studying the properties of glycine decarboxylation were only incubated for maximal periods of 10 min. During the standard 10 min. incubation typical rates for glycine decarboxylation (apparent Km value 1mM) were 0.4 µmole $^{14}CO_2$. 10 min$^{-1}$ mg mitochondrial protein$^{-1}$. In Table 1 the general properties of glycine decarboxylation has been recorded together with the rates of glycine

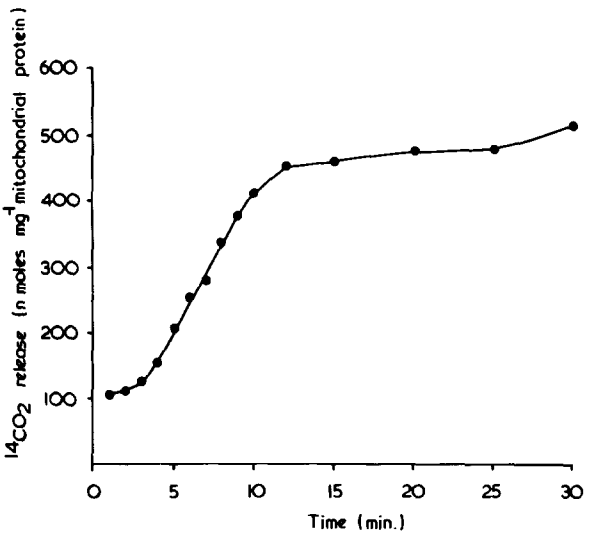


Figure 2

Time course of $CO_2$ evolution from glycine by spinach leaf mitochondria

Incubation medium (2.0 ml) as described in Fig. 1. Approximately 0.5 mg mitochondrial protein was added. Reaction was started by the addition of 5mM glycine containing 0.15 µCi [1-$^{14}$C] glycine as described in Materials and Methods.
The reaction was stopped at the times indicated.

dependent oxygen uptake. It is readily apparent from Table 1A that there is a direct relationship between these two processes since both are unaffected by the addition of co-factors such as tetrahydrofolate, pyridoxal phosphate or $NAD^+$ and are stimulated to a similar extent by ADP or FCCP. It is of interest to note that the addition of 2.0mM oxaloacetate stimulated glycine decarboxylation activity 3-fold and had a powerful transient inhibitory activity on glycine dependent oxygen uptake. It seems likely that the NADH produced as a result of glycine decarboxylation is freely available for re-oxidation by oxaloacetate in the presence of mitochondrial malate dehydrogenase, rather than being oxidised by the electron transport chain. This may explain the apparent 'second system' of glycine decarboxylation claimed by Woo and Osmond [8]. As expected, the electron transport inhibitors

TABLE 1

General Properties of Glycine Decarboxylation in Spinach leaf Mitochondria

| Conditions | $CO_2$ evolved nmoles.min$^{-1}$ mg protein$^{-1}$ | Oxygen Uptake nmoles. min$^{-1}$ mg protein$^{-1}$ |
|---|---|---|
| A | | |
| Complete | 33.7 (100) | 38 (100) |
| + 0.05mM Tetrahydrofolate | 30.6 (91) | 38 (100) |
| + 0.15mM pyridoxal phosphate | 33.3 (99) | 38 (100) |
| + 0.15mM thiamine pyrophosphate | 27.8 (82) | 38 (100) |
| + 1mM $NAD^+$ | 27.0 (80) | 38 (100) |
| + 2.5mM $NAD^+$ | 36.0 (107) | 40 (105) |
| + 2.5mM ADP | 97.2 (288) | 96 (253) |
| + 2.5 µM FCCP | 66.3 (198) | 96 (253) |
| + 2.0mM oxaloacetate | 112.2 (333) | 19 (50) |
| B | | |
| Complete | 33.0 (100) | 36 (100) |
| + 20 µM rotenone | 8.3 (25) | 30 (83) |
| + 1µg/ml antimycin A | 1.6 (5) | 9 (25) |
| + 1mM KCN | 7.2 (22) | 15 (42) |
| + 12mM INH | 19.5 (59) | 25 (69) |
| + 20mM INH | 11.1 (34) | 15 (42) |
| + 0.5mM PHMS | 31.0 (94) | 36 (100) |

Values measured are the mean of 4 experiments.

Complete incubation medium contained 300mM Mannitol, 5mM $MgCl_2$, 10mM KCl, 10mM phosphate buffer pH 7.2 and 0.1% BSA. Oxygen uptake was monitored during State 4 in the presence of 5mM glycine. For $^{14}CO_2$ studies, the reaction was started by the addition of 5mM glycine containing 0.15 µCi [1-$^{14}$C] glycine as described in Materials and Methods. The numbers in parentheses refer to percentage of control.

antimycin A, rotenone, and cyanide strongly inhibited glycine decarboxylation (Table 1B). INH (isonicotinyl hydrazide), a specific inhibitor of serine synthesis from glycine *in vivo* [19] inhibited both glycine decarboxylation and glycine dependent oxygen uptake to a similar extent with an apparent half maximal inhibition of 15mM. The glycollate oxidase inhibitor, αPHMS [20] however had no effect on either $^{14}CO_2$ release or oxygen uptake. Preliminary experiments suggest that glycine dependent oxygen uptake and glycine decarboxylase are specific for mitochondria in leaf tissues and furthermore are only associated with plants possessing photorespiratory activity in contrast to the results of Kisaki *et al* [4]. For example the rate of $^{14}CO_2$ release by intact etiolated mung bean hypocotyl mitochondria (photorespiration absent) is 0.1.8 nmoles $min.^{-1}$ mg $protein^{-1}$.

Discussion: An important point which has emerged from the experiments outlined in this communication is that intact spinach leaf mitochondria can oxidise glycine as a substrate and that the oxidation is coupled to ATP synthesis. Furthermore the evidence presented in Table 1 suggests that in spinach leaf mitochondria glycine-dependent oxygen uptake and the decarboxylase reaction are directly linked since both processes are inhibited by electron transport inhibitors and by INH, and are stimulated by ADP and uncoupling agents. It is suggested that glycine decarboxylase is located within the mitochondrial matrix, generating NADH, which in turn is oxidised endogenously *via* the electron transport chain. Presumably the oxidation associated with this decarboxylase activity proceeds through the internal pool of $NAD^+$ since all 3 sites of phosphorylation are involved (see Fig. 1). Hence addition of external $NAD^+$ does not stimulate the reaction. The coupling of glycine oxidation to 3 phosphorylation sites is particularly significant since it indicates that during rapid photorespiration considerable amounts of ATP are synthesised outside of the chloroplast. Photorespiration has frequently been regarded as an energy wasteful process.

Further evidence confirming the proposed location of glycine decarboxy-

lase was obtained by the use of rotenone which partially inhibited both the oxidation and decarboxylation processes and decreased the ADP/O ratios to 2. Rotenone is a specific inhibitor of the oxidation of $NAD^+$-linked substrates, although only partial inhibition is normally observed with plant mitochondria [14,21,22]. It would therefore appear that in the presence of rotenone reducing equivalents enter the chain *via* a dehydrogenase which bypasses the first phosphorylation site. The exact location of this dehydrogenase is under investigation.

Oxaloacetate readily enters plant mitochondria and exerts a powerful but transient inhibition of the rate of oxidation of most Krebs cycle dehydrogenases [23]. A similar effect on glycine oxidation was found in the present work. It is of interest to note that this inhibition was accompanied by a strong stimulation of glycine decarboxylation. This result is consistent with our suggestion that the glycine oxidising and decarboxylating system is located within the inner membrane of the mitochondrion. Presumably for efficient operation of the reaction the oxidising and decarboxylating system must be closely situated together in the inner membrane as is the case with other dehydrogenation complexes (see 14). We are currently attempting to isolate the system as an enzyme complex.

Acknowledgements

This work is supported by a grant from the Rank Prize Research Funds. A.L.M. is a Rank Prize Research Fellow.

References

1. Tolbert, N.E. (1971) Ann. Rev. Plant Physiol. 22, 45-74.
2. Halliwell, B. (1977) Prog. Biophys. Mol. Biol. In the press.
3. Zelitch, I. (1975) Ann. Rev. Biochem. 44, 123-45.
4. Kisaki, T., Yoshida, N. and Imai, A. (1971) Plant Cell Physiol. 12, 267-73.
5. Bird, I.F., Cornelius, M.J., Keys, A.J. and Whittingham, C.P. (1972) Phytochemistry 11, 1587-94.
6. Bird, I.F., Cornelius, M.J., Keys, A.J. and Whittingham, C.P. (1972) Biochem. J. 128, 191-192.
7. Collins, N., Brown, R.H. and Merrett, M.J. (1975) Biochem. J. 150, 373-377.
8. Woo, K.C. and Osmond, C.B. (1976) Aust. J. Plant Physiol., 3, 772-785.
9. Douce, R., Moore, A.L. and Neuberger, M. (1977) Plant Physiol. In the press.
0. Estabrook, R.W. (1967) Methods in Enzymol. 10, 41-47.

11. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem. 193, 265-275.
12. Arnon, D.I. (1949) Plant Physiol. 24, 1-15.
13. Lilley, McC. R., Fitzgerald, M.P., Rienits, K.G. and Walker, D.A. (1975) New Phytol. 75, 1-10.
14. Palmer, J.M. (1976) Ann. Rev. Plant Physiol. 27, 133-157.
15. Yoshida, T. and Kikuchi, G. (1970) Arch. Biochem. Biophys. 139, 380-392.
16. Motokawa, Y. and Kikuchi, G. (1971) Arch. Biochem. Biophys. 146, 461-466.
17. Romani, R.J. and Ozelkok, S. (1973) Plant Physiol, 51, 702-707.
18. Bonner, W.D. Jr., (1973) in Phytochem. Vol. 3 p.221. Van Nostrand, N.Y.
19. Pritchard, G.G., Griffin, W.J. and Whittingham, C.P. (1962) J. Exp. Bot., 13, 176-184.
20. Zelitch, I. (1972) Plant Physiol. 50, 109-113.
21. Coleman, J.O.D. and Palmer, J.M. (1972) Eur. J. Biochem. 26, 499-509.
22. Day, D.A. and Wiskich, J.T. (1974) Plant Physiol. 53, 104-109.
23. Douce, R. and Bonner, W.D. Jr., (1972) Biochem. Biophys. Res. Commun. 49, 619-624.