GLYCINE DECARBOXYLASE ACTIVITY IN PLANT TISSUES MEASURED BY A RAPID ASSAY TECHNIQUE

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1. Introduction

Photorespiration is a metabolic pathway which results in a light-dependent uptake of oxygen and release of previously fixed CO_2 and occurs almost exclusively in algae and the leaves of higher plants which fix CO_2 entirely by the Calvin cycle [1–10]. It is generally agreed that the majority of CO_2 is released when glycine is converted to serine, although additional CO_2 is released by the oxidation of glyoxylate and formate [2,3,8,10]. Glycine decarboxylase (EC 2.1.2.10) in conjunction with serine methylhydroxy transferase (EC 2.1.2.1) catalyses the conversion of glycine to serine releasing one molecule of CO_2 and NH_3 [11–15].

Glycine + tetrahydrofolate + NAD^+ →

N^5N^{10} methylene tetrahydrofolate

+ NH_3 + CO_2 + $NADH_2$

In mammalian tissues, the enzyme system is considered to be composed of four protein components involving pyridoxal phosphate, tetrahydrofolate and a protein exhibiting lipoamide dehydrogenase activity [14,15]. Recent evidence, in plant tissues, indicates that the oxidative decarboxylation of glycine, in isolated mitochondria, is associated with the synthesis of ATP [16–19].

Abbreviations: DCPIP, 2, 6, dichlorophenol-indophenol; FCCP, carbony cyanide *p*-trifluoromethoxyphenyl hydrazone; PHMS, 2-pyridylhydroxymethane sulphonic acid; OAA, oxaloacetate; NEM, *N*-ethylmaleimide

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The release of $^{14}CO_2$ following incubation with labelled substrates is routinely used as an assay for decarboxylase activity. Although such an assay is reasonably accurate, it has the disadvantage of being time consuming, not continuous and most importantly apparently dependent upon mitochondrial integrity [17,19]. It is therefore difficult to assess glycine decarboxylase (and hence photorespiratory activity) in crude homogenates.

Here, a rapid, continuous and simple assay for glycine decarboxylase is described which utilises the ability of this enzyme to reduce (possibly indirectly) 2,6 dichlorophenol-indophenol. Such an assay system works well in both crude homogenates as well as in intact mitochondria. The effects of various respiratory and photorespiratory inhibitors on the assay are described. A preliminary account of this assay has been presented [21].

2. Materials and methods

2.1. *Materials*

All reagents were of the highest quality commercially available. Spinach leaves were obtained from New Covent Garden, London. Maize, barley, green mung bean and French bean leaves were obtained from our own greenhouse. Etiolated leaves and hypocotyls were obtained from 7–14-day-old plants grown at 25°C and in the dark. DCPIP, isonicotinyl hydrazide, dicoumarol, rotenone antimycin-A and FCCP were obtained from Sigma. Glycidate was a gift from Dr R. Baxter, Shell Res. and PHMS was obtained from Aldrich Chemical Co.

2.2. Methods

Fractionation of leaf tissues either into crude homogenates or mitochondria was done essentially as in [22]. Intact and purified mitochondria were prepared as in [22,23]. All operations were done at 0–4°C.

The release of $^{14}CO_2$ from [1-^{14}C]glycine was monitored as in [19]. Mitochondrial oxygen consumption was measured polarographically using a Hansatech oxygen electrode (King's Lynn, England) in a 2.0 ml closed cell in a medium containing 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer (pH 7.2) and 0.1% bovine serum albumin.

In this technique glycine decarboxylase was assayed spectrophotometrically in a reaction medium as above with the further addition of 90 μM DCPIP and ~50–200 μg protein in 2.5 ml final vol. The reaction is started by the addition of 10 mM glycine and is followed by recording continuously the reduction of DCPIP at 600 nm (E_{600} = 19.1 $mM^{-1} . cm^{-1}$).

The concentration of other reagents added during the course of reduction is as indicated. Solutions of DCPIP, in water were made up daily. Protein was estimated by a modification of the Lowry procedure [24] and chlorophyll was measured according to [25]. Mitochondrial protein was estimated as in [18,23].

3. Results

The method involves the coupling of the oxidation of NADH, generated by glycine decarboxylation, to the reduction of an electron acceptor such as DCPIP. This dye was used, in preference of ferricyanide, merely because of its higher extinction coefficient rendering it a more sensitive method. It is apparent from fig.1 that the reduction of the dye is dependent upon glycine addition and is linear until full reduction is achieved. With mitochondrial preparations and in the absence of glycine, the blank rate of DCPIP reduction is low (4–6 nmol . min^{-1} . mg $protein^{-1}$) but was found to be significant with crude homogenates (see later). The reaction is linearly dependent upon protein concentration, the amount chosen being so selected that the absorbance decreases ~30–50% of the chart width in 15–30 s, thus permitting measurement of the initial velocity. A double reciprical plot of rate of ΔA against DCPIP concentration indicates that the app. K_m for DCPIP is ~84 μM. Phenazine methosulphate was not required as a catalyst.

Table 1 shows a comparison of $^{14}CO_2$ release, from

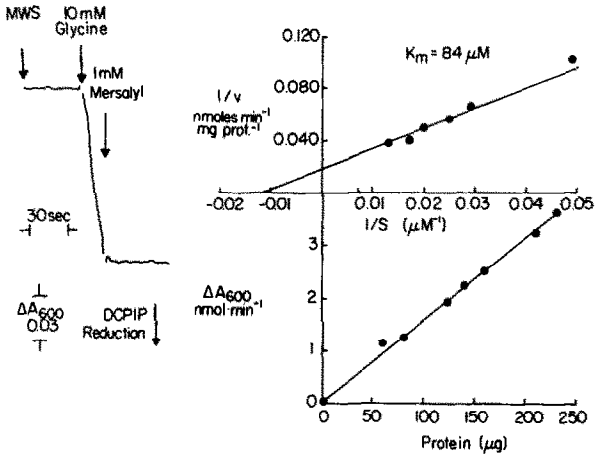

Fig.1. Glycine-dependent DCPIP reduction in spinach mitochondria. Assays were done in 2.5 ml total vol. reaction medium containing 90 μM DCPIP and ~0.06–0.1 mg mitochondrial protein except when varied as indicated. The reaction is initiated by the addition of 10 mM glycine and DCPIP reduction monitored at 600 nm, initial rates being measured. Additions were made as indicated.

10 mM [1-^{14}C]glycine, with the rate of glycine-dependent DCPIP reduction in spinach leaf mitochondria. Mitochondria were incubated for 5 min prior to analysis of the $^{14}CO_2$ released [19]. It can be seen that the rate of DCPIP reduction is similar to that observed with the ^{14}C assay although ~1/20th of the protein was required. Under comparable conditions, the rate of glycine-dependent oxygen consumption by spinach mitochondria was 19 nmol . min^{-1} . mg $protein^{-1}$.

Table 1
A comparison of $^{14}CO_2$ release with DCPIP reduction in spinach mitochondria

Addition	$^{14}CO_2$ release (nmol . min^{-1} . mg $protein^{-1}$)	DCPIP reduction (nmol . min^{-1} . mg $protein^{-1}$)
None	33.8	39.5
ADP 2.5 mM	69.3	39.4
FCCP 1 μM	66.1	37.1
OAA 1 mM	105.3	1.2
Antimycin A 1 μg/ml	18.4	36.5
Rotenone 20 μM	25.6	39.2

Assays were carried out as described in the text. For $^{14}CO_2$ studies, the reaction was started by the addition of 10 mM glycine (0.15 μCi [1-^{14}C]glycine) and monitored after 5 min. DCPIP reduction was followed at 600 nm. Values measured are the mean of 4 expt and are corrected for the blank rates. About 1.2 mg and 0.06 mg mitochondrial protein was used in the ^{14}C and DCPIP assays, respectively

Table 2
Effect of inhibitors on the rate of DCPIP reduction in spinach mitochondria

Addition	DCPIP reduction (nmol . min^{-1} . mg protein^{-1})	% Inhibition
None	39.5	0
KCN 1 mM	4.0	90
NEM 100 μM	0	100
Mersalyl 25 μM	0	100
Isonicotinylhydrazide 10 mM	26.3	36
Glycidate 2.5 mM	19.3	44
Dicoumarol 100 μM	28.8	27

Conditions were as in table 1. About 0.06 mg mitochondrial protein was used

ADP at 2.5 mM stimulated $^{14}CO_2$ release ~2-fold but had no effect on DCPIP reduction. Similar results were obtained with the uncoupling agent, FCCP. The inhibitory effects of rotenone and antimycin A, site 1 and 2 electron transport inhibitors, on $^{14}CO_2$ release and the lack of any effect on DCPIP reduction suggest that the ^{14}C assay depends upon respiratory chain activity to regenerate the NAD^+ whereas DCPIP interacts prior to the electron transport chain. Hence DCPIP reduction is not affected by compounds which stimulate or inhibit the mitochondrial respiratory chain. Oxaloacetate at 1 mM, however, has a marked stimulatory effect on $^{14}CO_2$ release, whilst potently inhibiting DCPIP reduction. Even at 10 μM oxaloacetate, a 100% inhibition of DCPIP reduction was observed. It seems likely, as suggested [19] that oxaloacetate stimulates $^{14}CO_2$ release by re-oxidising NADH via malate dehydrogenase. Its strong inhibitory effect on DCPIP reduction, however, indicates that the dye may well not interact with the decarboxylase directly.

Table 3
Assay of various plant tissue homogenates for glycine decarboxylase activities

Plant tissue	DCPIP reduction (nmol . min^{-1} . mg total protein^{-1})
Spinach (no addition)	162
Spinach + 40 μM dicoumarol	23
Spinach + 40 μM dicoumarol + 10 mM glycine	40
French bean hypocotyls (etiolated)	1.5
French bean hypocotyls (green)	1.2
French bean leaves (etiolated)	1.6
French bean leaves (green)	17.6
Maize leaves	0.5
Barley leaves	10.5

Crude homogenates were prepared as in [22]. DCPIP assay as in section 2. The values indicated for French bean, barley and maize leaves are corrected for blank rate in the presence of 40 μM dicoumarol

In table 2, we have investigated the effect of various inhibitors on glycine-dependent DCPIP reduction. The mercurials, mersalyl and *N*-ethylmaleimide, potently inhibit dye reduction, suggesting the involvement of sulphhydryl groups as essential components of the enzyme reaction mechanism. The strong inhibition by cyanide suggests that its effect is not solely as an electron transport inhibitor, as previously proposed, but may interact directly with the decarboxylase, possibly with disulphide moieties. High concentrations of dicoumarol, have relatively little effect on DCPIP reduction in contrast to its potent inhibitory properties on NADH–DCPIP reductase (diaphorase) [26]. The photorespiratory inhibitors [10] isonicotinylhydrazide and glycidate are only partially effective at inhibiting DCPIP reduction, whereas αPHMS, a glycolate oxidase inhibitor is without effect It would thus seem that glycine-dependent DCPIP reduction is a reasonable marker for glycine decarboxylase activity and that the dye interacts at a level other than the mitochondrial electron transport chain, or NADH–DCPIP reductase enzymes. The assay was therefore used to assess glycine decarboxylase activity in crude homogenates of several plant tissues. As indicated earlier, crude homogenates are complicated by the presence of a number of enzymes capable of oxidising NADH. Thus in the absence of glycine a considerable blank DCPIP reduction rate is apparent. Since this rate is significantly larger than the decarboxylase rate alone (150–200 nmol . min^{-1} . mg total protein^{-1}), a number of controls are required. We have found from a

series of experiments using spinach crude homogenates and mitochondria that a reliable estimate of the decarboxylase can be obtained from a subtraction of the blank rate observed in the presence of glycine. Alternatively, the inclusion of dicoumarol, to inhibit NADH–DCPIP reductases has been found to be particularly effective in distinguishing glycine–DCPIP reductase activity from any other DCPIP-reducing enzymes. For instance, in table 3, it can be seen that in the absence of glycine, the blank rate of DCPIP reduction in spinach crude homogenates is 162 nmol . min^{-1} . mg total $protein^{-1}$. This rate however, was found to be very sensitive to 40 μM dicoumarol (86% inhibited), a concentration which has little effect on glycine–DCPIP reductase activity in intact mitochondria (10% inhibition at 40 μM). Indeed, the subsequent addition of 10 mM glycine stimulated DCPIP reduction 74%. Thus it is possible, using this technique, to estimate glycine decarboxylase activity in crude homogenates without interference from other contaminating DCPIP reductase enzymes. Such a technique was used to estimate glycine decarboxylase in crude homogenates of French bean, maize and barley. Note that although decarboxylase activity is detectable in crude homogenates of French bean green leaves, it was not found in etiolated leaves or green hypocotyls. This result suggests that the enzyme may be light induced. Of particular interest is the finding that there is apparently little glycine decarboxylase activity in maize leaves, a C_4 tissue. This may be due to either insignificant amounts of the decarboxylase (in comparison to C_3 plants) or alternatively may merely reflect the difficulty of breakage and release of the bundle sheath cells in C_4 leaves.

4. Discussion

This report has utilised the reduction of DCPIP to develop a rapid and continuous assay for glycine decarboxylase.

The problem with artificial electron acceptors in an assay is the fact that they react at more than one site. However, the lack of an effect by antimycin and rotenone on DCPIP reduction indicates that the dye is not reacting with the mitochondrial respiratory chain (or electron transfer complexes). Further the insensitivity to dicoumarol suggests reduction is not by an NADH–DCPIP reductase [26]. The inhibition by mercurials, particularly *N*-ethylmaleimide and cyanide, suggests that the dye is reduced by a direct re-oxidation of a reduced flavin of the decarboxylase since NADH dehydrogenase is insensitive to these inhibitors [27]. The sensitivity to oxaloacetate does, however, argue against this suggestion. In order to account for this observation it must be proposed that the DCPIP interacts with the reduced flavin of NADH dehydrogenase rather than directly with the decarboxylase. Thus in the presence of oxaloacetate and malate dehydrogenase, NADH is re-oxidised thus preventing reduction of the dehydrogenase and its re-oxidation by DCPIP. Nevertheless, these data demonstrate that the assay apparently monitors glycine decarboxylase activity and can be applied to crude homogenates as well as to mitochondrial preparations. It may therefore prove particularly valuable for rapidly scanning tissues for photorespiratory activity, especially as it works effectively at 20-times lower protein concentrations than the ^{14}C-assay. Preliminary results suggest that it may be of use in the isolation and purification of the decarboxylase. Under these conditions a direct interaction of the dye with flavin of the decarboxylase may occur.

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