Measurement of *in vitro* glutamate synthase activity using an oxygen-evolving reconstituted chloroplast system

Evidence for enzyme activation by Mg^{2+} and 2-oxoglutarate

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1. INTRODUCTION

It is now evident that the major pathway for ammonia assimilation in plants involves the chloroplast enzyme, glutamate synthase (ECI4.7.1) [1]. Despite its importance however, measurement of the in vitro activity of this enzyme, in the past, has been time-consuming and somewhat complicated, involving the determination of the reaction product glutamate [2–4]. In order to allow for a more rapid and indeed more sensitive measurement of the in vitro activity of ferredoxin-dependent glutamate synthase, we have developed a reconstituted chloroplast system which exhibits GOGAT-dependent O_2 evolution. Such systems have been used to study individual enzymes and enzyme sequences of the Benson–Calvin cycle [5–7]; however, this is the first report of GOGAT-dependent O_2 evolution in a reconstituted chloroplast system. Furthermore, through the use of this assay system we have discovered regulatory characteristics not previously reported for this enzyme.

Abbreviations: GOGAT, glutamate synthase; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; BSA, bovine serum albumin; RuBP, ribulose 1,5-bisphosphate; 2-OG, 2-oxoglutarate; Chl, chlorophyll

2. MATERIALS AND METHODS

Pea seedlings (*Pisum sativum* var. Massey Gem) were grown in trays of vermiculite in a glasshouse for 10–13 days. All biochemicals were purchased from Sigma. Spinach ferredoxin was prepared as in [8].

Chloroplasts were isolated as in [9]. The chloroplast extract and envelope-free fractions were prepared essentially as in [5] by resuspending the chloroplast pellet in 5.5 ml of a solution containing 1/25 dilution of the standard reaction medium (330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes–NaOH (pH 7.6)) and 5 mM dithiothreitol, all adjusted to pH 7.6 with NaOH. After stirring for 2 min at 2°C the suspension was centrifuged for 10 min at $14000 \times g$. The supernatant (chloroplast extract) was collected and the pellet resuspended in 40 ml of the same medium and repelleted by centrifuging for 10 min at $14000 \times g$. This pellet was then resuspended in a small volume (2–3 ml) of full-strength reaction medium.

Oxygen evolution was measured in a Rank O_2 electrode maintained at 25°C and illuminated with a 150 W tungsten halogen projector lamp giving a light intensity of 550 $\mu E.m^{-2}.s^{-1}$ at the centre of the vessel. A standard reaction mixture contained envelope-free chloroplasts (200 μg chlorophyll), chloroplast extract (50–200 μl), 330 mM sorbitol,

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2 mM EDTA, 1 mM $MnCl_2$, 10 mM $MgCl_2$, 50 mM Hepes–NaOH, 1 mM NH_4Cl, 600 units of catalase, and 200 μg of spinach ferredoxin in a final volume of 2 ml at pH 7.6.

Chlorophyll was determined from 80% acetone extract as in [10].

3. RESULTS

The light-dependent evolution of O_2 by a reconstituted chloroplast system in the presence of 2-OG and glutamine is shown in fig. 1. It can be seen that no O_2 evolution occurs in the absence of either substrate, and that evolution is inhibited by azaserine, a specific inhibitor of the GOGAT enzyme [2] and by DCMU which inhibits photosynthetic electron transport. The rate of O_2 evolution was insensitive to the addition of methionine sulfoximine (not shown) indicating that the NH_4Cl present in the assay system, acted to uncouple electron transport only, and did not act together with the glutamate produced in the GOGAT reaction, to initiate any glutamine synthetase activity. The rate of O_2 evolution achieved on a per mg chlorophyll basis in the reconstituted system was dependent on the amount of chloroplast extract added (fig. 2). In the majority of experiments suboptimal levels of chloroplast extract were used to ensure that the supply of reducing equivalents from the chlorophyll fraction, did not limit the rate of GOGAT catalysed O_2 evolution, and consequently, the rates given in the other figures do not represent the maximum rates of O_2 evolution which could have been achieved using this reconstituted system.

The dependence of the reconstituted chloroplast system on added ferredoxin is illustrated in fig. 3. The K_m for ferredoxin was 0.9 μM which compares favourably to the value of 2 μM as estimated in [2] for their enzyme. Some background activity was

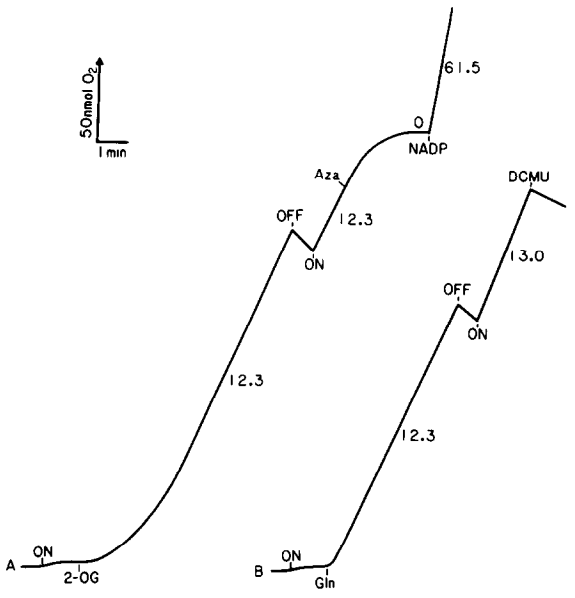

Fig. 1. GOGAT-dependent O_2 evolution in a reconstituted chloroplast system. Both traces contained 125 μl of chloroplast extract as well as: (A) 4 mM glutamine; (B) 1 mM 2-OG. Additions: (A) 2-OG, 1 mM; Azaserine, 0.5 mM; NADP, 0.16 mM; (B) glutamine, 4 mM; DCMU, 50 μM. Rates beside the curves represent μmol O_2 evolved.mg $chl^{-1}.h^{-1}$.

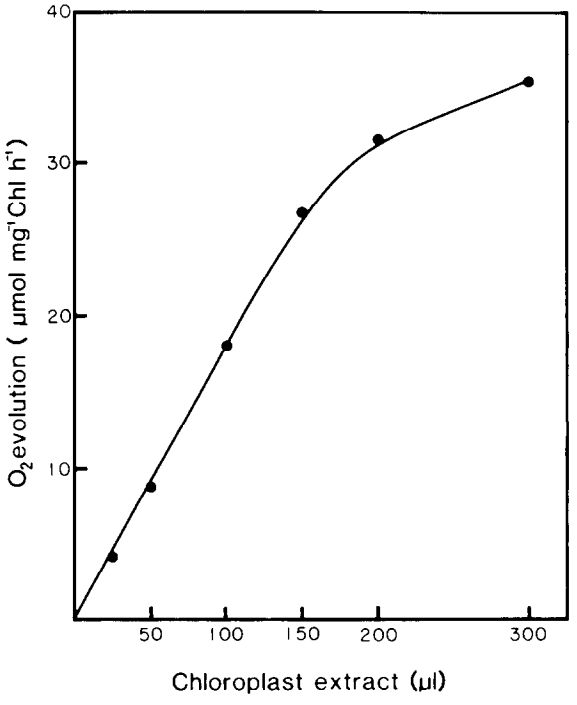

Fig. 2. Effect of chloroplast extract on the rate of GOGAT-dependent O_2 evolution in a reconstituted chloroplast system. Assays contained 4 mM glutamine and 2 mM 2-oxoglutarate and the reaction mixtures were pre-incubated with both substrates and chloroplast extract for 1 min in the dark prior to initiating O_2 evolution with light.

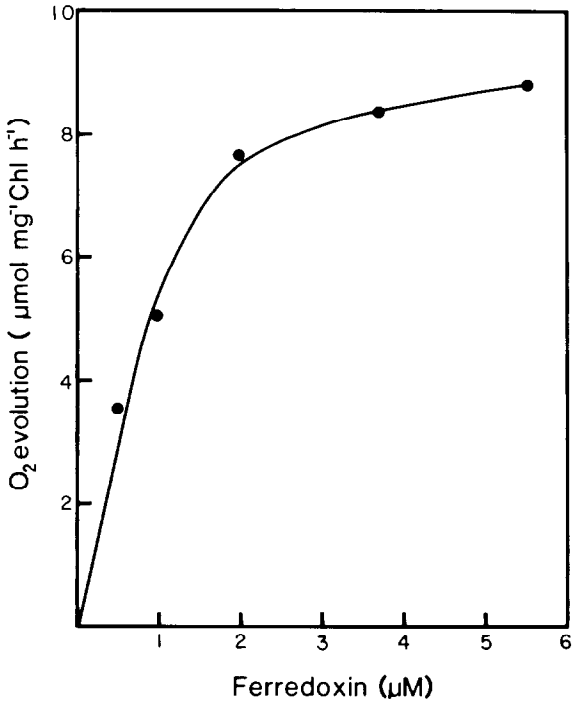

Fig. 3. Effect of spinach ferredoxin on the rate of GOGAT-dependent O_2 evolution in a reconstituted chloroplast system. Assays contained glutamine, 4 mM; 2-OG 2 mM and 50 µl of chloroplast extract. The reaction mixtures were pre-incubated for 1 min in the dark prior to initiating O_2 evolution with light.

achieved in the absence of added ferredoxin when larger volumes of chloroplast extract were employed in the reaction mixture, due to the presence of some ferredoxin in the chloroplast extract which was removed from the pea chloroplasts during the preparation of the reconstitution extracts.

GOGAT-dependent O_2 evolution in the reconstituted system demonstrated a broad pH optimum with a peak at pH 7.6 (fig. 4). The system also displayed an absolute requirement for Mg^{2+}, which could not be attributed to a requirement of the photosynthetic electron-transport chain for this ion, as demonstrated by the high rate of NADP-dependent O_2 evolution in the absence of added $MgCl_2$ (fig. 5). No GOGAT-dependent O_2 evolution was observed in the absence of added $MgCl_2$. This requirement for Mg^{2+} could be replaced to a certain extent by Ca^{2+} and Mn^{2+} but not by K^+ or Na^+ (table 1), showing the effect to be specific for a divalent cation.

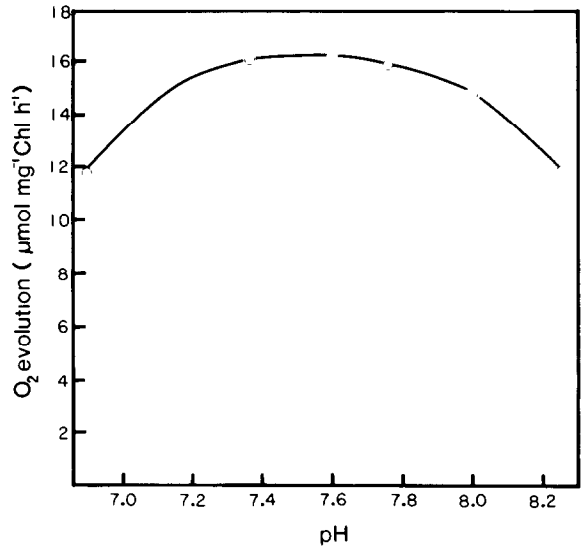

Fig. 4. Effect of pH on the rate of GOGAT-dependent O_2 evolution in a reconstituted chloroplast system. Standard assay contained glutamine, 5 mM; 2-oxoglutarate, 2.5 mM and 100 µl of chloroplast extract. The reaction mixtures were pre-incubated for 1 min in the dark prior to initiating O_2 evolution with light.

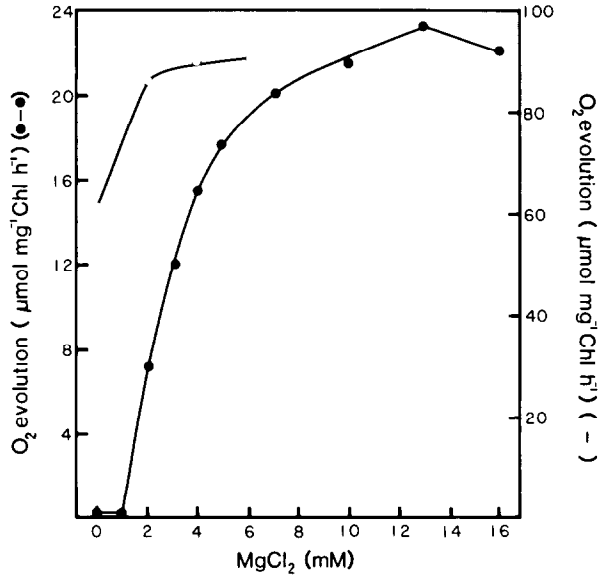

Fig. 5. Effect of [$MgCl_2$] on the rate of GOGAT- and NADP-dependent O_2 evolution in a reconstituted chloroplast system. Standard GOGAT assay contained glutamine, 4 mM; 2-OG, 2 mM and 125 µl of chloroplast extract. Assay for NADP-dependent O_2 evolution was as for GOGAT system, except for chlorophyll, 100 µg; ferredoxin, 125 µg; NADP, 1.0 mM. Reaction mixtures were pre-incubated for 1 min in the dark prior to initiating O_2 evolution with light.

Table 1

Effect of various inorganic salts on the rate of GOGAT-dependent O_2 evolution in a reconstituted chloroplast system

Salt	O_2 Evolution (μmol.mg chl^{-1}.h^{-1})
$MgCl_2$	15.2
$CaCl_2$	12.3
$MnCl_2$	9.9
KCl	0.0
NaCl	0.0

Standard assay contained glutamine, 4 mM; 2-OG 2 mM, and 150 μl of chloroplast extract. The various salts were added to 10 mM after incubation of the reaction mixture in the light for 1 min

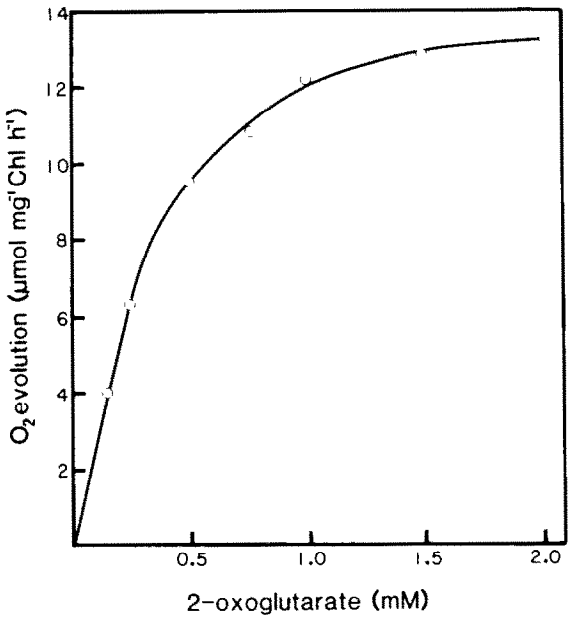

Fig. 6. Effect of [2-OG] on the rate of GOGAT-dependent O_2 evolution in a reconstituted chloroplast system. Standard assay contained glutamine, 4 mM and 125 μl of chloroplast extract. The reaction mixtures were pre-incubated for 1 min in the light prior to initiating O_2 evolution with 2-OG.

As shown in fig. 1 (trace A), a lag period is observed between the time of 2-OG addition to the reaction mixture and the achievement of the maximal rate of O_2 evolution. This did not occur, however, if the GOGAT enzyme was pre-incubated with 2-OG, prior to the initiation of O_2 evolution with glutamine (trace B, fig. 1) or with light, when both substrates were present (not shown). As this may indicate some form of allosteric interaction between the GOGAT enzyme and 2-OG, the effect of varying concentrations of this substrate, on the rate of O_2 evolution was investigated. While the results (fig. 6) tend to indicate a normal Michaelis–Menten type relationship between substrate concentration and reaction rate, it should be noted that the rates plotted are in fact the steady state rates achieved following the lag period, and that this lag period varied depending on the concentration of 2-OG present; e.g., 4 min at 0.15 mM 2-OG compared to 2 min at 2.0 mM 2-OG. The length of the lag was found to be independent of the glutamine concentration (not shown). The K_m for 2-OG was 0.27 mM, however, this may be somewhat of an overestimation, since the reaction rates measured at low [2-OG] (<0.5 mM) were found to markedly increase (e.g., 4.0–5.8 μmol.mg chl^{-1}.h^{-1} for 0.15 mM 2-OG) on a further addition of the same concentration of 2-OG to the reaction mixture, following the full utilization of the first 2-OG addition.

4. DISCUSSION

In [3] it was shown for the first time that GOGAT activity could be linked in vitro to the light-mediated generation of reduced ferredoxin by thylakoid membranes. However, as with previous assays for this enzyme, the major limitation in their system was the lack of a rapid and simple technique for the measurement of reaction rate. Our results show that this problem can be overcome through the use of a standard reconstituted chloroplast system as developed originally in [12], in which GOGAT activity can be measured directly from the rate of light-dependent O_2 evolution.

The absolute requirement for activity of the GOGAT enzyme for divalent cations and in particular Mg^{2+}, is in direct contrast to the results of previous studies which have found these cations to have an inhibitory effect on GOGAT activity [2,11]. This is however, a characteristic which has also been reported for a number of other chloroplast enzymes including RuBP carboxylase [13],

and fructose-1,6-diphosphatase [14] and has been proposed as a mechanism for the regulation of these enzymes, through light-dependent alterations in the [Mg^{2+}] in the chloroplast stroma [14,15]. The activation of the GOGAT enzyme by its substrate, 2-OG, as evidenced by the lag in activity if this substrate is not first pre-incubated with the enzyme, also shows a close parallel to the interaction of the RuBP carboxylase enzyme with its substrate, CO_2. In [13] it was shown that RuBP carboxylase forms an active ternary complex (enzyme–CO_2–Mg^{2+}), in which CO_2 first binds to the enzyme in a slow, rate-determining step, followed by the rapid binding of Mg^{2+}. Consequently, unless the enzyme is pre-incubated with CO_2, a lag in activity will result, the length of which is found to be proportional to the concentration of CO_2 present. Our results show that a similar relationship appears to exist between GOGAT, 2-OG and Mg^{2+}; with the interaction between the enzyme and 2-OG being the slow, rate-limiting step, while Mg^{2+}-binding was rapid and did not result in a lag in activity on addition (not shown). Further work is required to identify potential allosteric sites on the GOGAT enzyme to which 2-OG and Mg^{2+} may be required to bind to ensure full activation of the enzyme, and which may indeed represent important points of regulation of the photorespiratory cycle.

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