Polarographic Study of Ammonia Assimilation by Isolated Chloroplasts

Received for publication March 25, 1977 and in revised form June 2, 1977

JOHN W. ANDERSON² AND JAMES DONE³
Department of Biochemistry, Rothamsted Experimental Station, Harpenden, Herts. AL52JQ, England

ABSTRACT

Illuminated pea (Pisum sativum) chloroplasts catalyze (ammonia plus α-ketoglutarate [α-KG]-dependent O₂) evolution at rates which are commensurate with other estimates of the flux of assimilated nitrogen (mean of eight determinations, 8.3 μmole per mg chlorophyll per hour, SD 2.4). The reaction was usually initiated with 1 mM ammonia after preincubating chloroplasts in the presence of α-KG, ADP, pyrophosphate, and MgCl₂.

Progressive increases in ammonia concentration gave Vₘₐₓ/2 at 0.2 mM (approximately) and Vₘₐₓ at about 1 mM. Higher concentrations were inhibitory; at 7 mM the rate was again about Vₘₐₓ/2. The highest ratio of O₂ evolved per mol of ammonia supplied was 0.36.

The (ammonia plus α-KG)-dependent reaction was inhibited by methionine sulfoximine, azaserine, and aspartate in the presence of amino-oxoacetate but not by amino-oxoacetate alone and not by L-glutamate. The rate of O₂ evolution in the presence of 1 mM ammonia and 2.5 mM α-KG was increased only slightly by addition of 5 mM glutamine. Similarly, the rate of O₂ evolution in the presence of 5 mM glutamine and 2.5 mM α-KG was increased only slightly by addition of 1 mM ammonia.

The results are attributed to the incorporation of ammonia via glutamine synthetase and reductive transamination of the glutamate formed by photosynthetically coupled glutamate synthase using α-KG as the amino acceptor. Several lines of evidence rule out the possibility that photosynthetically coupled glutamate dehydrogenase is involved.

It has long been known that glutamate is an early product of ammonia assimilation in plants (3, 5). It now seems to be generally agreed that chloroplasts are the main site of reduction of nitrite to ammonia in leaf tissue (13, 15). Glutamate dehydrogenase (NADPH-specific), glutamine synthetase, and glutamate synthase have been reported in chloroplasts suggesting that these organelles might also be an important site of ammonia assimilation.

Illuminated chloroplasts catalyze the formation of glutamate from α-ketoglutarate (7) in the absence of exogenous ammonia. Ammonia (10 mM) had no significant effect on the reaction but the effect of lower concentrations was not reported. The reaction was attributed to photosynthetically coupled glutamate dehydrogenase (NADPH-specific). This enzyme is bound to the lamellae of Vicia faba chloroplasts (11). Glutamate dehydrogenase might account for ammonia assimilation in chloroplasts although the rate of glutamate formation (0.44 μmol mg Chl⁻¹ hr⁻¹) is low in comparison with the nitrite assimilation rates of chloroplasts of 6 to 12 μmol mg Chl⁻¹ hr⁻¹ (13) and 8 to 9 μmol mg Chl⁻¹ hr⁻¹ (15). Further, the enzyme from Lactuca sativa chloroplasts has a low affinity for ammonia (10).

Mitchell and Stocking (18) have recently shown that illuminated pea chloroplasts catalyze the formation of [¹⁴C]glutamate from [¹⁴C]glutamate in the presence of 1 mM ammonia at initial rates of 21 to 26 μmol mg Chl⁻¹ hr⁻¹. This suggests that glutamine synthetase, an enzyme known to be associated with chloroplasts (19, 23) and which has a high affinity for ammonia (20), might fulfill an important role in ammonia assimilation. The recent discovery that glutamate synthase is also associated with chloroplasts (9) means that N from the amide group of glutamine can be transferred to α-ketoglutarate to form glutamate and that there is an alternative mechanism for N assimilation (16).

Certain earlier experiments on the metabolite-labeling patterns of leaves following application of [¹⁴N]H₄ and [¹⁴C]O₂ have recently been reinterpreted in favor of the glutamine synthetase/glutamate synthase pathway (16). While it has been shown that these two enzymes are operative in isolated chloroplasts (9, 18), it has not yet been shown that ammonia assimilation is directly linked to glutamate synthase activity and electron transport and no comparative study of the rates of light-dependent ammonia assimilation by the glutamate dehydrogenase and glutamine synthetase/glutamate synthase pathways in illuminated chloroplasts has been reported.

Chloroplasts exhibit photosynthetically coupled glutamate synthase activity which can be measured as (glutamine plus α-ketoglutarate)-dependent O₂ evolution (1). If the initiation of ammonia incorporation is the formation of glutamine from glutamate catalyzed by glutamine synthetase then the glutamine so formed should be metabolized by photosynthetically coupled glutamate synthase. In this event it should be possible to demonstrate (ammonia α-ketoglutarate)-dependent O₂ evolution although such activity could also be attributed to photosynthetically coupled glutamate dehydrogenase activity. In this paper we report a study of (ammonia plus α-ketoglutarate)-dependent O₂ evolution which we attribute to glutamine synthetase and photosynthetically coupled glutamate synthase activities.

MATERIALS AND METHODS

Chloroplasts were prepared from pea seedlings (Pisum sativum cv. Feltham First). Oxygen evolution, chloroplast intactness, and Chl were measured as described previously (1). All acidic substrates were adjusted to pH 7 with KOH.

---

¹ This work was conducted while the authors were on sabbatical leave from their respective universities.
² Permanent address: Botany Department, La Trobe University, Bundoora, Vic. 3083, Australia.
³ Permanent address: Biochemistry Department, University of Sydney, Sydney, N.S.W. 2006, Australia.
‡ Definition: the term "photosynthetically coupled" is used throughout this paper to refer to the generation of a reductant by photosystems I and II (e.g. reduced ferredoxin or NADPH) which is used as a substrate in the associated reaction.
The routine procedure for measuring (ammonia plus \(\alpha\)-ketoglutarate)-dependent \(O_2\) evolution was to illuminate chloroplasts (300–450 \(\mu\)g Chl) in 2 ml of incubation medium (1) supplemented with 2.5 mM \(\alpha\)-ketoglutarate, 4.5 mM ADP, 5 mM PPI, 10 mM \(\delta\)-glyceraldehyde, and 10 mM MgCl\(_2\) (final concentration 11 mM). After 10 min, \(O_2\) evolution was initiated by addition of 2 or 4 \(\mu\)mol of NH\(_4\)Cl (1 or 2 mM, respectively); 1 mM ammonia was preferred as 2 mM was slightly inhibitory. If the total \(O_2\) evolution in experiments initiated with 1 mM ammonia exceeded 0.4 \(\mu\)mol (per 2 ml), it was necessary to add an additional 1 \(\mu\)mol of ammonia to ensure that this substrate did not become rate-limiting.

Glutamine plus \(\alpha\)-ketoglutarate-dependent \(O_2\) evolution was measured as described for (ammonia plus \(\alpha\)-ketoglutarate)-dependent activity except that \(O_2\) evolution was initiated with 10 \(\mu\)mol of L-glutamine (5 mM). This procedure produces slightly lower rates than those we described previously (1) but it was adopted to allow a comparison of the ammonia- and glutamine-dependent activities under similar experimental conditions.

**RESULTS**

Chloroplasts which were preincubated in the light with \(\alpha\)-ketoglutarate, ADP, PPI, and MgCl\(_2\) for 10 min evolved \(O_2\) when supplied with 2 mM ammonia (Fig. 1A). Some chloroplast preparations, and chloroplasts which were stored at 0 C for 1 to 2.5 hr, frequently exhibited a time lag of up to 2.5 min before \(O_2\) evolution commenced. The rate of \(O_2\) evolution usually increased to a maximum over a period of 0.3 to 10 min and thereafter remained constant. The reaction did not proceed in the absence of \(\alpha\)-ketoglutarate or in the dark. When chloroplasts were preincubated with 2 mM ammonia instead of \(\alpha\)-ketoglutarate, and 2.5 mM \(\alpha\)-ketoglutarate added after 3 min, \(O_2\) evolution commenced after a time lag of 6 min and the rate gradually increased to a maximum (Fig. 1B). Taken collectively, these experiments establish that additions of both ammonia and \(\alpha\)-ketoglutarate are required for \(O_2\) evolution. Since \(O_2\) evolution was inhibited by 1 \(\mu\)M DCMU and it did not occur in the dark, we concluded that the reaction(s) was dependent on photosynthetic electron transport.

The effect of time of preincubation on the \(O_2\) evolution response following addition of ammonia and \(\alpha\)-ketoglutarate, respectively, was examined in more detail for one chloroplast preparation. When ammonia was supplied to chloroplasts which had been preincubated with \(\alpha\)-ketoglutarate for 10 min or more, \(O_2\) evolution reached maximum rate in less than 0.5 min; but if ammonia was supplied before 10 min, there was a time lag before \(O_2\) evolution commenced and the maximum rate was not attained until about 10 min had elapsed. The highest rates of \(O_2\) evolution were obtained if ammonia was added after a minimum preincubation time of 10 min (Fig. 2). While all chloroplast preparations were not examined in such detail it was noticed that the time of preincubation needed for elimination of the lag varied, according to the preparation, from 2 to 14 min. When chloroplasts were preincubated with ammonia for various times prior to adding \(\alpha\)-ketoglutarate, there was a time lag before \(O_2\) evolution commenced. The time lag, the maximum rate, and the time taken to achieve maximum rate decreased with increasing time of preincubation. Even when the preincubation time was 17 min there was still a lag of 3 min before \(O_2\) evolution commenced and a period of 6 min before attaining maximum rate.

Oxygen evolution was not detected following the addition of ammonia to reaction mixtures lacking any one of the following compounds: ADP, MgCl\(_2\), or PPI (Fig. 3). The time lag for commencement of \(O_2\) evolution was much longer for the incubation mixture initially lacking PPI (8 min) than for those lacking MgCl\(_2\) (3.3 min) or ADP (3.3 min).

The reaction was specific for \(\alpha\)-ketoglutarate. Oxygen evolu-
tion did not occur when α-ketoglutarate was replaced by 10 mM l-glutamate, 10 mM glyoxylate, or 10 mM pyruvate. Glutamate (2–10 mM) neither affected (ammonia plus α-ketoglutarate)-dependent O₂ evolution nor the length of the preincubation period required before ammonia initiated an immediate commencement of O₂ evolution.

The rate of O₂ evolution increased at first very rapidly when ammonia concentration was progressively increased up to 1 mM (Fig. 4A) and then decreased to about half the maximum rate at 7 mM; only at 17 mM did ammonia completely inhibit O₂ evolution.

The effect of limiting amounts of ammonia was studied in relation to O₂ evolution. A small addition of ammonia (0.25 or 0.5 μmol/2 ml) initiated O₂ evolution in the usual manner but the rate of O₂ evolution soon decreased and finally ceased; O₂ evolution recommenced when a further addition was made (Fig. 5). This procedure could be repeated several times. Higher rates of O₂ evolution occurred after the second or subsequent additions of ammonia and shorter times were required to attain the maximum rate. The ratio of O₂ evolved/mol of ammonia supplied was calculated for each addition of ammonia (Table I); the highest ratios (0.34, 0.36) were obtained with those chloroplast preparations which required the shortest preincubation times before responding to the first addition of ammonia. Similar experiments with α-ketoglutarate were not feasible in view of the long time lag before O₂ evolution commenced.

Azaserine inhibits glutamate synthase of pea chloroplasts (1). In the present study we found that 0.1 to 0.25 mM azaserine also completely inhibited (ammonia plus α-ketoglutarate)-dependent O₂ evolution suggesting that O₂ evolution is dependent on the formation of glutamine which is then metabolized via photosynthetically coupled glutamate synthase. Methionine sulfoximine also inhibited (ammonia plus α-ketoglutarate)-dependent O₂ evolution by isolated pea chloroplasts; 1 mM, 1.5 mM, and 2.5 mM methionine sulfoximine caused 15%, 31%, and 100% inhibition of O₂ evolution, respectively. When ammonia plus α-ketoglutarate-dependent O₂ evolution was inhibited by methionine sulfoximine, addition of glutamine caused a resumption of O₂ evolution at a rate similar to that before addition of the inhibitor (Fig. 5).

Freshly prepared chloroplasts catalyze (ammonia plus α-ketoglutarate) and (glutamine plus α-ketoglutarate)-dependent O₂ evolution at approximately similar rates under the experimental conditions described under “Materials and Methods.” Oxygen evolution in the presence of 1 mM ammonia and 2.5 mM α-ketoglutarate was enhanced 22% by addition of 5 mM glutamine. Similarly, O₂ evolution in the presence of 5 mM glutamine and 2.5 mM α-ketoglutarate was enhanced 41% by addition of 1 mM ammonia. In both cases the rates of O₂ evolution in the presence of both glutamine and ammonia were less than the arithmetic sum of the rates with either glutamine or ammonia alone.

Addition of 5 mM amino-oxyacetate, an inhibitor of transaminases (14), to the standard incubation mixture did not affect O₂ evolution but subsequent addition of aspartate caused a marked inhibition (Fig. 6B). In the absence of amino-oxyacetate, aspartate (2.5 mM) enhanced the rate of O₂ evolution (Fig. 6A).

**DISCUSSION**

The characteristics of (ammonia plus α-ketoglutarate)-dependent O₂ evolution are, to a large extent, consistent with the assimilation of ammonia in chloroplasts by way of the following series of reactions:

\[
\text{ADP} + \text{Pi} \xrightarrow{hv} \text{ATP} \quad \text{(I)}
\]

\[
\text{NH}_3 + \text{glutamate} + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + \text{Pi} \quad \text{(II)}
\]

\[
\text{H}_2\text{O} + 2 \text{ferredoxin (ox)} \xrightarrow{hv} \frac{1}{2}\text{O}_2 + 2 \text{ferredoxin (red)} \quad \text{(III)}
\]

\[
\text{Glutamine} + \alpha\text{-ketoglutarate} + 2 \text{ferredoxin (red)} \rightarrow 2 \text{glutamate} + 2 \text{ferredoxin (ox)} \quad \text{(IV)}
\]

\[
\text{H}_2\text{O} + \text{NH}_3 + \alpha\text{-ketoglutarate} \rightarrow \frac{1}{2}\text{O}_2 + \text{glutamate} \quad \text{(V)}
\]
AMMONIA ASSIMILATION BY CHLOROPLASTS

FIG. 5. Effect of limiting amounts of ammonia on O₃ evolution in presence of 2.5 mM α-ketoglutarate. O₂ evolution was measured in standard reaction mixtures and additions were made as shown. Values beside curves represent rate of O₂ evolution in μmol mg Chl⁻¹ hr⁻¹. Chl concentration, 200 μg ml⁻¹; chloroplast intactness, 66%; volume, 1.7 ml.

Table I. Molar ratios of oxygen evolved following the addition of small amounts of ammonia in the presence of 2.5 mM α-ketoglutarate.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Preincubation time</th>
<th>Intactness</th>
<th>Chlorophyll conc.</th>
<th>Number of addition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>%</td>
<td>mg ml⁻¹</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>1</td>
<td>4.3</td>
<td>72</td>
<td>225</td>
<td>0.13 0.20 0.21 0.19</td>
</tr>
<tr>
<td>2</td>
<td>10.2</td>
<td>74</td>
<td>200</td>
<td>0.20 0.24 0.23 0.17</td>
</tr>
<tr>
<td>3</td>
<td>9.0</td>
<td>74</td>
<td>150</td>
<td>0.20 0.25 0.22 0.21</td>
</tr>
<tr>
<td>4</td>
<td>4.9</td>
<td>66</td>
<td>200</td>
<td>0.34 0.36</td>
</tr>
</tbody>
</table>

An effect on any one of these reactions can be indirectly monitored by the observed O₂ evolution. Reaction I requires light (Fig. 1), and the decreased rate of O₂ evolution at 2 to 17 mM ammonia (Fig. 4B) may be due to uncoupling of phosphorylation from electron transport (8). Reaction II, catalyzed by glutamine synthetase, has a high affinity for ammonia (Fig. 4A) and is inhibited by methionine sulfoximine (ref. 16, Fig. 5). Reaction III requires light (Fig. 1) and is inhibited by DCMU. Reaction IV, catalyzed by glutamate synthase, is inhibited by azaserine. Glutamate, a substrate for reaction II, was not required to initiate O₂ evolution. Once O₂ evolution commences, the system becomes self-sustaining with respect to glutamate (reaction IV). Our results are consistent with the studies of Mitchell and Stocking (18) on glutamine synthesis (reactions I and II) and our previous results on (glutamine plus α-ketoglutarate)-dependent O₂ evolution (reactions III and IV) (1).

The requirements for ADP and PPI, which inhibit plant glutamine synthetase (21, 25), and for MgCl₂ are not fully understood. We suspect that they are required for reaction I (4, 22) and that a favorable concentration of MgCl₂ is also required for reaction II (20). While Mitchell and Stocking (18) reported that chloroplasts catalyzed glutamine synthesis in the absence of ADP, Robinson and Wiskich (22) found that chloroplasts from young peas required catalytic amounts of ADP or ATP to promote maximum rates of CO₂-dependent O₂ evolution whereas those from more mature peas did not. PPI has been used as a slow source of Pi for CO₂ fixation by isolated chloroplasts (4).

Inhibition by methionine sulfoximine of O₂ evolution from (ammonia plus α-ketoglutarate), which could be overcome by adding glutamine, provided strong evidence that the pathway leading to O₂ evolution involves the synthesis of glutamine, which is catalyzed by glutamate synthetase, and that glutamine formation precedes the O₂ evolution reaction involving glutamate synthase. Whereas we found that net O₂ evolution rates following addition of substrates for both of the photosynthetically coupled enzymes (nitrite reductase and glutamate synthase) were equal to the sum of the rates when the respective substrates were added alone (1), there was no similar relationship for glutamate synthase and glutamine synthetase. We conclude that ammonia and glutamine compete for the reaction product which is coupled to O₂ evolution (i.e. photosynthetically coupled glutamate synthase).

The effects of aspartate and amino-oxyacetate on (ammonia plus α-ketoglutarate)-dependent O₂ evolution (Fig. 6) are simi-
lar to those described for (glutamine plus $\alpha$-ketoglutarate)-dependent activity (1) and they provide further evidence for the involvement of glutamine as an intermediate in (ammonia plus $\alpha$-ketoglutarate)-dependent $O_2$ evolution. Here again, it seems that amino-oxyacetate inhibits the formation of oxaloacetate by transamination of aspartate with the $\alpha$-ketoglutarate so that the oxaloacetate formed from aspartate is no longer reduced by the very active photosynthetically coupled malate dehydrogenase present in the preparations. Aspartate also apparently participates in a reaction which competes with glutamate synthase for glutamine (1).

Several of our observations indicate that photosynthetically coupled glutamate dehydrogenase is not involved in the observed (ammonia plus $\alpha$-ketoglutarate)-dependent $O_2$ evolution: the requirement for ADP, PPI, and MgCl$_2$; the function of glutamine as an intermediate; and the preparations of glutamate dehydrogenase (2, 16) our system was strongly inhibited by azaserine and methylene sulfonoxime.

The net sum of reactions I to IV (i.e., reaction V) predicts the evolution of 0.5 mol $O_2$/mol ammonia. The highest ratios determined by experiment were 0.36 and 0.34 (Table I). This implies that either ammonia and/or glutamine is metabolized by processes in addition to those described in reactions III and IV. Some evidence that glutamine is metabolized by other reactions in illuminated chloroplasts has been described previously (1). The highest molar ratios for ammonia are greater than those observed for glutamine (0.20 in the absence of arsenate) (1) and provide further evidence that glutamine synthesized from ammonia is more available to glutamine synthase than exogenous glutamine.

Some characteristics of the (ammonia plus $\alpha$-ketoglutarate)-dependent $O_2$ evolution from our preparations cannot be interpreted simply on the basis of reactions I to IV: the requirement for ADP and Pi, compounds which inhibit glutamine synthetase from pea leaves (21, 25); added glutamate had no effect although it inhibited glutamate synthase (1, 17); long preincubation periods were needed to eliminate a lag before ammonia would initiate $O_2$ evolution at maximum rate (Fig. 2); a corresponding lag occurred when $\alpha$-ketoglutarate was used to initiate the reaction which could not be eliminated by preincubation nor by addition of glutamine. We suspect that reconstituted chloroplast systems (24, 26) might provide a suitable means for investigations of these problems.

The effect of ammonia concentration on (ammonia plus $\alpha$-ketoglutarate)-dependent $O_2$ evolution (Fig. 4) is of considerable interest, particularly in relation to the common procedure where 2 to 4 mm ammonia is used to uncouple osmotically shocked chloroplasts, with ferricyanide as electron acceptor (12). The results in Figure 4 emphasize that 2 mm ammonia does not cause any appreciable inhibition of a metabolic activity dependent on both photos phosphorylation and electron transport in intact chloroplasts. However, 8 mm ammonia caused 57% inhibition of CO$_2$-dependent $O_2$ evolution by pea chloroplasts (22) and this value is in close agreement with that described for (ammonia plus $\alpha$-ketoglutarate)-dependent activity (Fig. 4, 56% inhibition at 8 mm).

The average rate of (ammonia plus $\alpha$-ketoglutarate)-dependent $O_2$ evolution was 8.3 $\mu$mol mg Chl$^{-1}$ hr$^{-1}$ (52.4 for eight independent preparations). Assuming a theoretical stoichiometry of $O_2$ evolution to ammonia consumption of 0.5 (reaction V), this represents the incorporation of 16.6 $\mu$mol of ammonia mg Chl$^{-1}$ hr$^{-1}$ or a N flux of 16.6 $\mu$g atoms of N mg Chl$^{-1}$ hr$^{-1}$. This value is in good agreement with the N flux of glutamate formation by glutamine synthetase (18) and glutamine metabolism by glutamate synthase (1) catalyzed by isolated pea chloroplasts of similar intactness to those used in the present study.

The results imply that the N flux of ammonia to glutamate involving reactions I to IV occurs at approximately the same rate as reactions (I and II) and reactions (III and IV) when measured independently. Our results show that the energy requirements for ammonia incorporation in chloroplasts can be readily supplied by the light reactions. The average rate of N flux (16.6 $\mu$g atoms mg Chl$^{-1}$ hr$^{-1}$) can be compared with a CO$_2$-dependent $O_2$ evolution rate (in the presence of ATP) for pea chloroplasts of 70 to 130 $\mu$mol mg Chl$^{-1}$ hr$^{-1}$ (22); this represents an average photosynthetically coupled C flux of 100 $\mu$g atoms mg Chl$^{-1}$ hr$^{-1}$. Isolated pea chloroplasts therefore have relative photosynthetically coupled C to N fluxes of 6:1 (atom/atom) whereas typical C to N ratios of plant material are 40:1 (atom/atom) (6). These considerations show that chloroplasts have the capacity to assimilate more ammonia relative to CO$_2$ than required to produce the typical C to N ratios of plant material.

Acknowledgment. — The cooperation and interest shown by D. A. Walker and by colleagues at Rothamsted Experimental Station are gratefully acknowledged.

LITERATURE CITED

3. CHIBNALL AC 1939 Protein Metabolism in the Plant. Yale University Press, New Haven pp 87-103
4. CROOKSIN W, CW BALDWIN, DA WALKER 1967 Some effects of inorganic phosphate on $O_2$ evolution by isolated chloroplasts. Biochim Biophys Acta 143: 614-624
8. GOOD NE 1960 Activation of the Hill reaction by amines. Biochim Biophys Acta 40: 502-517
11. LEVEN RM, PR KIRK 1968 An NADP-dependent glutamate dehydrogenase from chloroplasts of Vicia faba L. Biochim Biophys Acta 56: 685-690