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Evidence for the Glutamine Synthetase/Glutamate Synthase Pathway during the Photorespiratory Nitrogen Cycle in Spinach Leaves

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ABSTRACT

Spinach leaf (*Spinacia oleracea* L.) discs infiltrated with [^{15}N]glycine were incubated at 25°C in the light and in darkness for 0, 30, 60 and 90 minutes. The kinetics of ^{15}N-incorporation into glutamine, glutamate, asparagine, aspartate, and serine from [^{15}N]glycine was determined. At the beginning of the experiment, just after infiltration (0 min incubation) serine, and the amido-N of glutamine and asparagine were the only compounds significantly labeled in both light- and dark-treated leaf discs. Incorporation of ^{15}N-label into the other amino acids was observed at longer incubation time. The per cent ^{15}N-enrichment in all amino acids was found to increase with incubation. However, serine and the amido-N of glutamine remained the most highly labeled products in all treatments. The above pattern of ^{15}N-labeling suggests that glutamine synthetase was involved in the initial refixation of $^{15}NH_3$ derived from [^{15}N]glycine oxidation in spinach leaf discs.

The ^{15}N-enrichment of the amino-N of glutamine was found to increase rapidly from 0 to 19% during incubation in the light. There was a comparatively smaller increase (4–9%) in the ^{15}N-label of the amino-N of glutamine in tissue incubated in darkness. Furthermore the total flux of ^{15}N-label into each of the amino acids examined was found to be greater in tissue incubated in the light than those in the dark. The above evidence indicates the involvement of the glutamine synthetase/glutamate synthase pathway in the recycling of photorespiratory NH_3 during glycine oxidation in spinach leaves.

Under atmospheric conditions, the CO_2 released in photorespiration is believed to be derived from the conversion of glycine to serine in the mitochondria (3, 7, 18). In mitochondria isolated from spinach leaves, the conversion of glycine to serine also led to the stoichiometric release of CO_2 and NH_3 (6, 19). Evidently during photosynthesis at atmospheric conditions, the flux of this photorespiratory N in leaves of C_3 plants is substantial and would be expected to be equivalent to the rate of photorespiration. Evidence from *in vitro* studies (6, 15, 19), indicating that the NH_3 released during glycine oxidation in mitochondria is not refixed by GDH[2] within the mitochondria but rather by GS in the cytoplasm or chloroplast has led to the proposal that the GS/GOGAT pathway is involved in the refixation and recycling of this photorespiratory N. The isolation of mutants of *Arabidopsis thaliana* (L) Heynh deficient in GOGAT activity which could not survive in conditions that permit photorespiration (11) has provided direct evidence for the involvement of such a pathway in the photorespiratory N cycle. But the precise fate of the NH_3 released during glycine oxidation within the cell remains to be established.

In this study we have examined the pattern of the ^{15}N-incorporation in amino acids in spinach leaf discs infiltrated with [^{15}N] glycine. The evidence confirms the involvement of the GS/GOGAT pathway in the photorespiratory N cycle *in vivo*.

MATERIALS AND METHODS

Experimental. Spinach leaf (*Spinacia oleracea* L.) discs (0.5 cm, 1 g fresh weight) were placed in 50 ml Erlenmeyer flasks containing 10 ml of 20 mM K-phosphate (pH 7.5), and 10 mM [^{15}N]glycine (99% enriched, Stohler). Flasks containing leaf discs were vacuum infiltrated and the procedure repeated twice. The total time taken was 10 min. Flasks were then transferred to shaking water-bath (25°C) and incubation commenced under illumination (200 μE m^{-2} s^{-1}). For dark treatments the flasks were wrapped in two layers of aluminum foil. Flasks were removed at 0, 30, 60 and 90 min incubation. All treatments were carried out in triplicate. At the end of incubation, leaf discs were washed with 60 ml ice-cold K-phosphate (pH 7.5) solution and transferred immediately to 25 ml boiling water for 30 min. The aqueous extract was filtered and freeze dried.

Preparation of Amino Acid Fractions. The amino acid fractions were separated by ion-exchange chromatography. Initially, the freeze-dried extracts were resuspended in 4 ml water and loaded on to a column of Dowex 50W (H^+ form) ion-exchange resin. The amino acid fraction was eluted with 4 N NH_4OH. Subsequently, this amino acid fraction was separated into the acidic (glutamate and aspartate) and the neutral/basic amino acid fractions on Dowex 1 × 8 (acetate form) ion-exchange resin. A portion of the basic/neutral amino acid fraction was hydrolyzed in 2 N HCl at 100°C for 60 min to convert glutamine and asparagine to glutamate and aspartate, respectively. These two acidic amino acids were then separated from the other amino acids, as described above, and used for the determination of the ^{15}N-enrichment of the amino-N of the two amides from which they were derived. All the amino acid fractions were freeze dried. Amino acid content was determined by a Beckman 119 Amino Acid Autoanalyzer.

GC-MS Analyses. The amino acid fractions were resuspended in 50% ethanol. Aliquots were removed and dried under N_2. These samples were washed and dried twice initially with absolute

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[2] Abbreviations: GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; TMS, trimethylsilyl; BSTFA, *N,O-bis*-(trimethylsilyl)-trifluoroacetamine; TMCS, trimethylchlorosilane.

ethanol and then with methylene chloride. TMS-derivatives of the amino acids were prepared by heating the above fractions in the presence of acetonitrile and BSTFA/TMCS at 70°C for 15 min. These derivatives were than analyzed for ^{15}N-enrichment by GC-MS as previously described (1, 13). Briefly, the principle on which the method is based is that described by Bieman (2), where the natural isotope pattern of the specific fragment ion being measured is calculated using its elemental composition and the natural abundances of its constituent elements. Deviations from this natural abundance pattern are then determined from the relative heights of the peaks of the isotope cluster in the enriched sample and expressed as the percentage of molecules unlabeled (R) or labeled at one (R + 1) or two (R + 2) mass units above this. For ^{15}N-enrichments in amino acids with a single nitrogen R + 1 is the percentage of molecules labeled. For the amides glutamine and asparagine, R + 1 and R + 2 are the percentages of singly and doubly labeled species, respectively. Because as hydrolysis of the amides can be used to determine the amino-N label specifically, the amido-N label can then be calculated algebraically.

RESULTS

Figures 1 and 2 show the time course of ^{15}N-label incorporation into some amino acids in spinach leaf discs infiltrated with [^{15}N] glycine in the light and in the dark respectively. At the beginning of the experiment, just after infiltration for 10 min, only serine (18–19% labeled) and the amido-N of glutamine (6% labeled) and asparagine (3–5% labeled) are significantly labeled in both the light and dark treatments. There is no significant incorporation of ^{15}N-label in the amino-N of glutamine and asparagine nor in glutamate and aspartate. Evidently the conversion of [^{15}N]glycine to [^{15}N]serine and the subsequent assimilation of the released $^{15}NH_3$ into the amido-N of glutamine have occurred during the 10 min infiltration of the tissue with [^{15}N]glycine. The significant incorporation of the ^{15}N-label into the amido-N of glutamine but not into the amino-N of glutamine nor into glutamate during this period suggests that the released $^{15}NH_3$ has been refixed preferentially via GS (rather than GDH) activity.

During incubation, the ^{15}N-label in all the amino acids examined increases in both light and dark treated tissue. In all the treatments the ^{15}N-label in serine and the amido-N of glutamine remains the most highly labeled throughout the course of the experiment. After 90 min incubation, the ^{15}N-enrichment in serine has increased from 18% to 58% in the dark compared to an increase of from 19% to 32% in the light. Under the same conditions, the ^{15}N-enrichment of the amido-N of glutamine increases from 6% to 23% in both light and dark. In contrast, the ^{15}N-enrichment in glutamate and aspartate is only 14% and 11%, respectively, after 90 min of incubation in the light. The ^{15}N enrichment of the amino-N of glutamine increases rapidly (from 0%–19%) during incubation in the light (Fig. 1) compared to a relatively small change (from 4%–9%) in the ^{15}N-label of the amino-N of glutamine in tissue incubated in darkness (Fig. 2).

The amino acid content in the leaf tissue has been determined in order to estimate the total ^{15}N-incorporation into all of the amino acids examined. Figure 3 shows the time course of the total ^{15}N incorporation into serine, glutamine, glutamate, asparagine, and aspartate in tissue incubated in the light and in the dark. The data show that the total ^{15}N-incorporation into these amino acids is greatly increased in the light compared to the dark. The largest increase is observed in glutamate and aspartate.

DISCUSSION

In this study we have examined the pattern of the incorporation of ^{15}N-label into various amino acids in spinach leaf discs infiltrated with [^{15}N]glycine. The order of labeling suggests that (a) the nitrogen of serine is derived from glycine during glycine

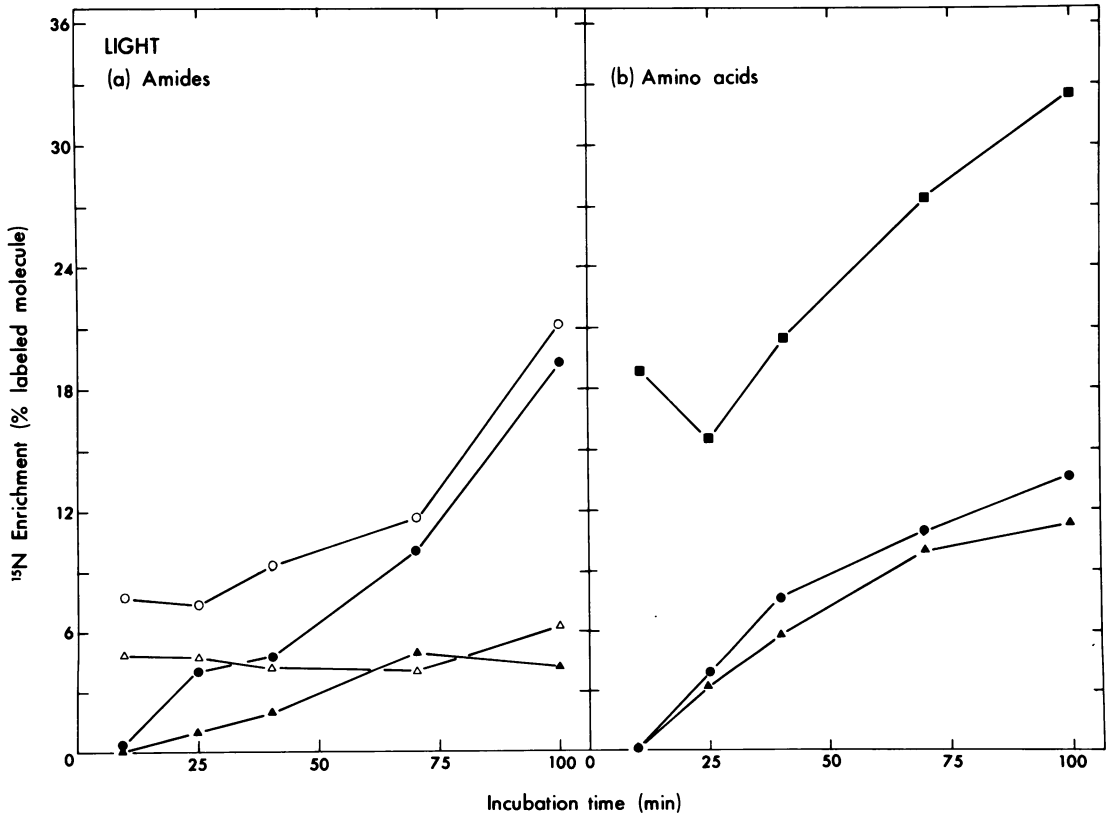

FIG. 1. Time course of ^{15}N-incorporation into amides and amino acids in spinach leaf discs infiltrated with [^{15}N]glycine and incubated in the light. The incubation time has been adjusted to include the 10 min taken for vacuum infiltration. Amides: amino-N (●) and amido-N (○) of glutamine; amino-N (▲) and amido-N (△) of asparagine. Amino acids: (●), glutamate; (▲), aspartate; (■), serine.

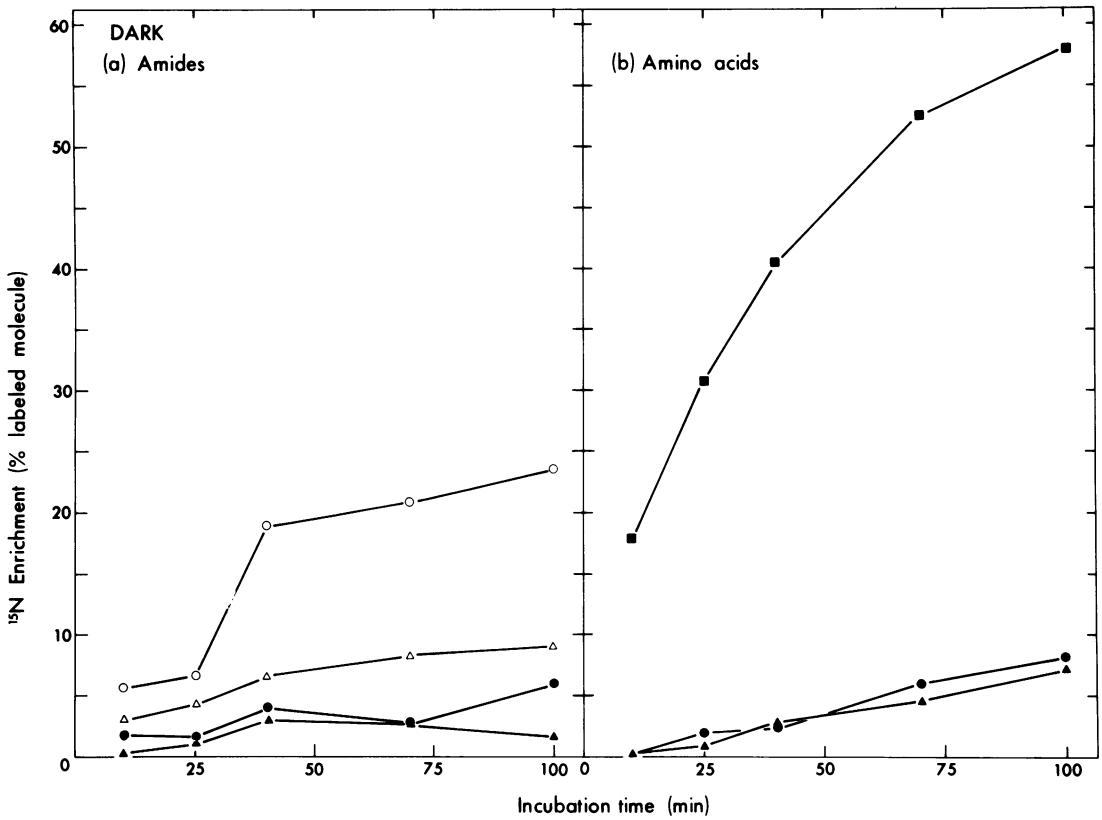

FIG. 2. Time course of ^{15}N-incorporation into amides and amino acids in spinach leaf discs infiltrated with [^{15}N]glycine and incubated in the dark. The incubation time has been adjusted to include the 10 min taken for vacuum infiltration. Amides: amino-N (●) and amido-N (○) of glutamine; amino-N (▲) and amido-N (△) of asparagine. Amino acids: (●), glutamate; (▲), aspartate; (■), serine.

oxidation in the mitochondria, (b) the nitrogen released as NH_3 during glycine oxidation is incorporated directly into the amido-N of glutamine via glutamine synthetase, and (c) there is a light-dependent reductive transfer of this amido-N of glutamine to glutamate and the amino-N of glutamine via GOGAT activity (8).

The initial processes, *i.e.* (a) and (b) can proceed in the dark as well as in the light. This is not surprising, inasmuch as the cytosolic GS (16) would enable refixation of the released $^{15}NH_3$ to proceed in the dark. Ito *et al.* (5) have also observed substantial ^{15}N enrichment in the amido-N of glutamine in isolated spinach leaf cells incubated with $(^{15}NH_4)_2SO_4$ in darkness. The above evidence, however, does not distinguish whether the refixation of photorespiratory NH_3 by GS in the light takes place in the cytosol (7, 15) and/or in the chloroplast (17).

In the light, the considerable increase in the total ^{15}N flux through all the amino acids examined (Fig. 3) is correlated with a rapid rise in the ^{15}N-enrichment of the amino-N of glutamine (Fig. 1). The former, presumably, is due to increased activity of glycine decarboxylation *per se* (supported by an increased availability of keto acid acceptors, *e.g.* glyoxylate and α-ketoglutarate, generated during photosynthesis and photorespiration), whereas the latter would have involved ferredoxin-dependent GOGAT activity. This suggests that the increase in total N flux in the light is most likely linked to the increased N flux through the GS/GOGAT pathway. But the precise magnitude of the flux through this pathway is not known because the sizes of active and inactive pools of the various amino acids have not been determined in this study. However, the above evidence is consistent with evidence from studies with mutants of *Arabidopsis* deficient in GOGAT activity (11) and from studies with isolated leaf mitochondria (6, 15, 19). Together, all the evidence suggests that the GS/GOGAT pathway is the major route for the reassimilation and recycling of photorespiratory ammonia.

On the other hand, the substantial incorporation of ^{15}N-label into glutamate and aspartate in tissue incubated in the dark (Fig. 2) does not appear to involve this pathway. Ito *et al.* (5) have shown that the deamination of glutamine can occur in the dark. Under these conditions, the amido-N of glutamine so released could undergo reductive amination with α-ketoglutarate to form glutamate via GDH activity. But the concentration of NH_3 would have to be high for this reaction to proceed since GDH has a high K_m for NH_3 in the range of 5 to 100 mM (*cf.* 9). Furthermore, it has been demonstrated in isolated leaf mitochondria that GDH could synthesize glutamate only under nonphysiological conditions (4). Thus, although it is possible that a part of the ^{15}N-label observed in glutamate and aspartate in leaf discs incubated in the dark could still be derived via GDH as discussed above, it is more likely that the greater part of this ^{15}N-incorporation is due to the transfer of ^{15}N-label from the recently formed [^{15}N]serine (via [^{15}N]glycine oxidation), directly or indirectly, to glutamate and aspartate via transaminase activities (14) either in net synthesis or equilibration exchange.

In the light the amino group of glycine is likely to be formed from unlabeled glutamate during photorespiration. This would lead to a dilution of the ^{15}N-label of the glycine supplied exogenously and thus account for the lower ^{15}N-enrichment in serine observed in the light compared to the dark (Fig. 1 and 2). The ^{15}N-enrichment in the amido-N of glutamine is lower than that of serine in both light and dark treatments. This low value could be due, at least, to two factors: (a) the presence of inactive or alternative pool(s) of glutamine in the tissue, and (b) the isotopic dilution of $^{15}NH_3$ by endogenous pool(s) of NH_3.

Asparagine is thought to be formed by the transfer of the amido-N of glutamine to aspartate via asparagine synthetase activity (10, 12). In the present study, the rapid labeling of the

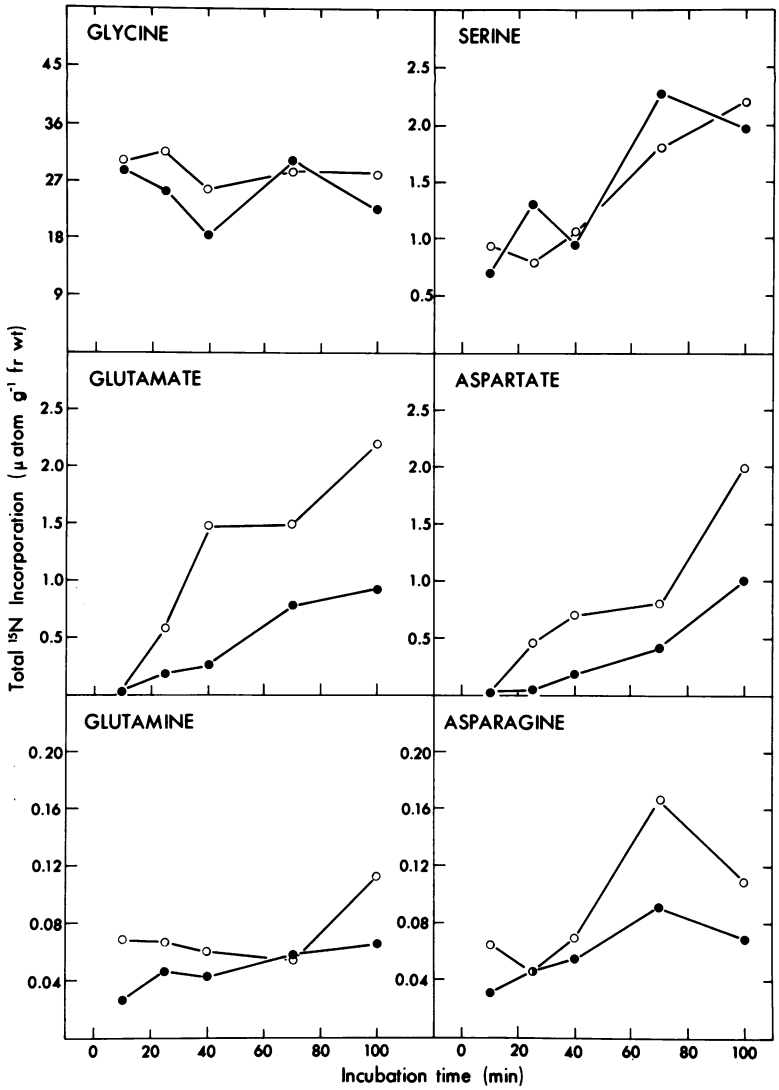

FIG. 3. Time course of total ^{15}N-incorporation (% enrichment × amino acid content) into amino acids in spinach leaf discs infiltrated with [^{15}N] glycine and incubated in the light (○) and in the dark (●). For amides, total ^{15}N-incorporation is the sum of the amido and amino-N labeling. The incubation time has been adjusted to include the 10 min taken for vacuum infiltration.

amido-N of asparagine in the light and in the dark (Figs. 1 and 2) suggests that such an amido-N transfer from glutamine probably occurs in spinach leaves. Furthermore, the steady increase of ^{15}N-label in the amino-N of asparagine during incubation in the light (Fig. 2) suggests that such transfer continues in the light.

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