Kinetics and Energetics of Light-driven Chloroplast Glutamine Synthesis1

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ABSTRACT
Chloroplasts contain the enzyme glutamine synthetase. Formation of glutamine by isolated chloroplasts is light-dependent and requires an intact outer envelope. Addition of exogenous glutamic acid, as well as nitrogen donors such as nitrate or ammonium, stimulate the synthesis of this amide. Photosynthetic generation of ATP satisfies the light requirement of glutamine synthesis. The process is supported by cyclic as well as noncyclic photophosphorylation.

The rapid passage of photosynthetically elaborated carbon into amino acids and protein in a number of species has aroused considerable interest in the chloroplast as an important cellular compartment of nitrogen assimilation (4, 5, 9, 14). Among the first compounds labeled by 14CO2 in algae and leaves in the light are alanine, aspartate, serine, glycine, and later glutamate (26, 27), suggesting that the necessary enzymes and precursor pools exist in the chloroplast for the synthesis of these amino acids. Isolated chloroplasts of higher plants have been shown to incorporate 14CO2 into alanine, aspartate, and glycine (25, 31).

During amino acid feeding experiments (25), neither glycine nor serine were found to be accumulated or metabolized in either the light or in darkness by in vitro preparations of spinach chloroplasts containing a large proportion of class I plastids. However, these preparations were able to take up 14C-glutamate and rapidly convert it to 14C-glutamine in the light, but not in darkness. This conversion took place to the same extent with or without cofactors, ATP, NH3, or NO3, indicating that the intact chloroplasts retained the enzymatic mechanism necessary to effect the transformation. The 14C from the added glutamate appeared only in glutamine. When the chloroplasts were swollen by osmotic shock, they lost their ability to synthesize glutamine (25).

Recent work in this laboratory has shown that chloroplasts contain the enzyme L-glutamate-ammonia ligase (ADP, EC 6.3.1.2), more commonly referred to as glutamine synthetase, which catalyzes the reaction:

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glutamate + NH_3 + ATP \rightarrow \text{glutamine} + \text{ADP} + \text{Pi}
\]

The cellular distribution and characterization of foliar glutamine synthetase will be reported in a subsequent article. This report describes the kinetics of the reaction and the manner by which chloroplast glutamine synthesis is coupled to photosynthetic energy sources.

MATERIALS AND METHODS

Plant Material. Seeds of dwarf pea (Pisum sativum L. cv. Progress No. 9) were germinated in a screened sand-peat-vermiculite preparation, 1:1:2 by volume, in 15.2-cm plastic pots. The seedlings were grown in a greenhouse and watered routinely with full strength Hoagland's solution. Each container was additionally fertilized with 100 ml of 18 g/l of NH4H2PO4, 5 days after planting. When a large number of leaves were fully expanded (14–18 days from planting) plants were destarched in the dark for 24 hr and then placed under low intensity light for 1 hr before harvest of leaves. Spinach was obtained from a local market, refrigerated moist in darkness overnight, and illuminated for 1 hr in water before isolation of chloroplasts.

Chloroplast Isolation Medium and Procedure. Chloroplast isolations were performed according to a series of modifications of the method of Jensen and Bassham (17). The original method requires three separate solutions, each containing 0.33 M sorbitol, 0.02 M NaNO3, 0.02 M NaEDTA, 0.002 M Na isoascorbate, 0.001 M MnCl2, 10−4 M MgCl2, and 5 × 10−4 M KH2PO4. In addition, the isolation medium, pH 6.1 (solution A), contains 0.05 M MES, and 0.02 M NaCl. The resuspension medium, pH 6.7 (solution B), contains 0.05 M HEPES, and 0.02 M NaCl, and the incubation medium, pH 7.6 (solution C), contains 0.05 M HEPES, and 0.005 M Na2PO4·10 H2O. Modifications of these media employed to protect the integrity of the labile pea chloroplasts included raising theionicity of the osmolytic, sorbitol, to 0.4 M in all three solutions, adding 0.50% (w/v) fatty acid-free BSA to the isolation medium, and omitting PPI from solution C because it created permeability (24) and solubility problems. In studies involving the uptake and assimilation of exogenous NH4+ or NO3− by isolated whole pea chloroplasts, NaNO3 was omitted from all solutions to eliminate adding any other external nitrogen source. All reagents were prepared CO2-free in order to avoid possible competition effects for ATP by Calvin cycle reactions.

A chloroplast isolation procedure was adopted which preserves a high percentage of plastids in an intact condition. Ten or 15 g of fully expanded leaves were harvested, washed, chilled, and presoaked in three volumes of solution A. Leaves were then rapidly sliced in the chilled medium over ice with an electric knife modified to fit a razor blade holder on each shortened shank. The rapid reciprocating motion of the blades ruptures leaf cells and releases cellular contents without localized heating and adverse shearing effects. The homogenizing vessel was a deep flexible plastic container. The crude homogenate was pressed through one layer of Miracloth and the filtrate was centrifuged at 9,000 g for 30 min. The pellet was gently resuspended
with a pipette in a volume of solution B sufficient to give chloroplasts equivalent to 25 μg of Chl/ml after filtration through one layer of tissue paper. The chloroplast resuspension was held in darkness over ice until assay.

**Incubation and Illumination Procedure.** L-Glutamic acid-4C (U), 270 mCi/mmmole, was supplied as a deoxygenated aqueous solution containing 2% ethanol (Amersham-Searle Corp., Arlington Heights, Ill. 60005). Radiochemical purity was estimated to be 98 to >99% by several analytical methods. In order to eliminate alcohol and make further purifications, the stock glutamic acid was applied to a Dowex-1 (Cl-) column at pH 7, washed with water, eluted with 0.01 M HCl, frozen, reduced to dryness by lyophilization, and redissolved in an appropriate volume of solution C.

Incubations were performed in round-bottomed tubes in an illuminated Warburg apparatus at 25 C and 2400 ft-c of incandescent light. The spectral flux density over the interval from 375 to 750 nm was 38,322 ergs cm⁻² sec⁻¹ as measured with an ISCO Model SR Spectroradiometer (Instrumentation Specialties Co., Lincoln, Neb. 68507). In experiments concerned with effects of far red light on glutamine synthesis, the illumination source consisted of three 375-w reflector flood lamps mounted 10.2 cm from the incubation tubes with a 0.32-cm thick acrylic far red cutoff filter (Westlake Plastics, Inc., Lenni, Pa.) and water bath intervening. This filter (FRF-700) permits only wavelengths greater than 700 nm to be transmitted. Under these conditions, the far red source has an irradiance of 20,400 ergs cm⁻² sec⁻¹ from 700 to 750 nm, whereas the unfiltered white light source produced only 8420 ergs cm⁻² sec⁻¹ over the same wavelength interval.

Reaction media contained variable concentrations of the following reagents in a final volume of 0.125 or 0.200 ml: "cold" carrier glutamic acid, NH₄Cl or NaN₃, and purified "C-glutamate (about 0.25 μCi), all in solution C, pH 7.6; 0.05 ml of glutamate solutions in solution B, pH 6.7; and inhibitors or cofactors in solution C to final volume.

Incubations were initiated by adding the chloroplast resuspension to the reaction medium. The reaction tube was gently shaken at 25 C for the duration of the incubation. The reaction was terminated by rapidly adding enough ice-cold stop solution to make a final volume of 1 ml. The stop solution, pH 7.0, consisted of 0.02 M imidazole, 0.03 M glutamine, and sufficient NaF to make a final concentration of 10⁻⁴ M (7).

After the reaction was terminated, a 0.1-ml aliquot of the mixture was added directly to a 0.5 × 5.5 cm Dowex-1 (Cl-) anion exchange column (200–400 mesh) from which the "fines" had been removed. The sample was chased by 1.9 ml of 0.01 M imidazole HCl 0.03 M glutamine, pH 7. Under these conditions, glutamic acid remains 100% bound to the resin while glutamine is 90 to 95% eluted (22). Two ml of eluate from each column was collected directly into a scintillation vial, 10 ml of Bray's solution (6) were added, and radioactivity was determined with a Packard Tri-Carb liquid scintillation counter. Quenching was corrected for by the method of internal standardization (33). Thus, the amount of radioactivity eluted from a column at pH 7 is a quantitative measurement of glutamine synthesized.

A quantitative determination of the integrity of isolated chloroplasts in a preparation was obtained by one of two different methods, depending upon experimental restrictions: light-dependent, NADP-stimulated O₂ evolution before and after plastids have been osmotically shocked (24), and a phase contrast microscopic method of counting class I (intact) and class II (broken) chloroplasts. Class I chloroplasts were assumed to be those which appear bright, refractile, opaque, and birefringent around their edge when viewed at 1000 magnifications, whereas class II were those which appear dark, swollen with visible grana, and are losing or have lost their birefringence (20).

Chlorophyll was determined according to the method of Arnon (1).

CCCP was obtained from Calbiochem, Los Angeles, Calif., salicylaldoxime was obtained from Eastman Kodak Co., Rochester, N.Y., and DCMU was obtained in a highly purified form from E. I. du Pont de Nemours and Co., Wilmington, Del.

**RESULTS**

A time course of pea plastid glutamine synthesis (Fig. 1) indicates that intact plastids convert glutamate to glutamine at a linear rate for about 12 min in the light under the given conditions, after which no further incorporation takes place. Although the stoichiometry of glutamine formation requires that each data point also represents glutamate taken up by the plastid, it is not known whether glutamine formed is equivalent to glutamate uptake. The initial rate of glutamine production by chloroplasts in Figure 1 is 21 μmoles mg Chl⁻¹ hr⁻¹. Since glutamine synthesis is intact plastid-dependent, and since the resuspended plastids were determined to be 54.6% intact, correction of this rate for a hypothetical preparation of completely intact plastids would become 39.2 μmoles mg Chl⁻¹ hr⁻¹.

The effect of NH₄⁺ concentration on light-driven glutamine synthesis was investigated in the presence of a high 50 μmoles of glutamate per ml. The results are summarized in Figure 2. The low but measurable amount of glutamine formed in the absence of exogenous NH₄⁺ clearly indicates the presence within the chloroplast of available nitrogen pools equivalent to amounts obtained with 10⁻⁴ to 10⁻³ M exogenous NH₄⁺. Glutamine synthesis by whole chloroplasts was stimulated by a NH₄⁺ concentration of 10⁻³ M, reached a maximum at 10⁻⁴ M NH₄⁺, decreased at NH₄⁺ levels above 10⁻⁴ M, and became maximally inhibited at 0.1 M NH₄⁺. The rate of glutamine synthesis

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**FIG. 1.** Time course of glutamine synthesis by isolated whole pea chloroplasts at 25 C and 2400 ft-c. The incubation mixture contained 6.25 μmoles of monosodium glutamate, 0.125 μmoles of NH₄Cl, 0.31 μCi of purified "C-glutamate, chloroplasts equivalent to 35.5 μg of Chl, and solution C to a final volume of 0.125 ml. Percentage of intact plastids was determined to be 54.6%.

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*Abbreviation: CCCP: carbonylcyanide-m-chlorophenylhydra-
by a preparation containing 71% intact plastids was 26 μmoles mg Chl⁻¹ hr⁻¹ with 10⁻⁴ M NH₄⁺. The corrected rate (for 100% intact plastids) would be 37 μmoles mg Chl⁻¹ hr⁻¹.

Once 10⁻⁴ M NH₄⁺ was established as optimal concentration for the intact plastid reaction, exogenous glutamate was then varied through a similar 5-fold order of magnitude concentration range, as shown in Figure 3. The observed small increase in glutamine synthesis with a large change in glutamate concentration indicates a significant permeability barrier to added glutamate. The synthesis mechanism did not saturate at any glutamate concentration used. However, the assay was standardized at 5 × 10⁻⁴ M glutamate for subsequent investigations.

![Graph](image1)

**Fig. 2.** Effect of NH₄⁺ concentration on glutamine synthesis by isolated whole pea chloroplasts in 8 min at 25 C and 2400 ft-c. The incubation mixture contained 6.25 μmoles of monosodium glutamate, 0.34 μCi of ¹⁴C-glutamate, a range of NH₄⁺ concentrations, chloroplasts equivalent to 25.2 μg of Chl, and solution C to a final volume of 0.125 ml. Percentage of intact plastids in the resuspension was determined to be 71%.

![Graph](image2)

**Fig. 3.** Effect of glutamic acid concentration on glutamine synthesis by isolated whole pea chloroplasts in 8 min at 25 C and 2400 ft-c. The incubation mixture contained 0.125 μmole of NH₄Cl, 0.30 μCi of purified ¹⁴C-glutamate, a range of monosodium glutamate concentrations, chloroplasts equivalent to 25.4 μg of Chl, and solution C to final volume of 0.125 ml. Percentage of intact plastids in the resuspension was determined to be 53.6%.

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**Table I. Effect of Light, Dark, and Exogenous Nitrogen on Glutamine Synthesis by Isolated Whole Pea Chloroplasts**

The incubation mixture contained 6.25 μmoles of monosodium glutamate, ±0.125 μmole of NaNO₂, 0.27 μCi of ¹⁴C-glutamate, chloroplasts equivalent to 8.15 μg of Chl, and solution C to a final volume of 0.125 ml. Incubations were performed for 8 min at 25 C with gentle shaking and ±2400 ft-c of incandescent light. The resuspension contained 60% intact plastids as determined by phase contrast light microscopy.

<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Dark (A)</th>
<th>Light (B)</th>
<th>Light-stimulated (B-A)</th>
<th>B-A for 100% Intact Plastids</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.23</td>
<td>1.84</td>
<td>1.61</td>
<td>2.69</td>
</tr>
<tr>
<td>10⁻³ M NH₄⁺</td>
<td>0.35</td>
<td>7.96</td>
<td>7.61</td>
<td>12.71</td>
</tr>
<tr>
<td>10⁻³ M NO₂⁻</td>
<td>0.27</td>
<td>4.27</td>
<td>4.00</td>
<td>6.67</td>
</tr>
</tbody>
</table>

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**Table II. Coupling oj Light-stimulated Glutamine Synthesis by Isolated Whole Spinach Chloroplasts to Cyclic and Noncyclic Photophosphorylation**

Each reaction mixture contained 10 μmoles of monosodium glutamate, 0.2 μmole of NH₄Cl, 0.323 μCi of purified ¹⁴C-glutamate, and chloroplasts equivalent to 23.5 μg of Chl in a final volume of 0.200 ml. In addition, certain treatments contained 0.04 nmole of DCMU, 0.04 μmole of salicylaldoxime, or 2 nmole of CCCP. Incubations were performed for 8 min at 25 C.

![Graph](image3)

**Fig. 4.** Effect of glutamic acid concentration on glutamine synthesis by isolated whole spinach chloroplasts in 8 min at 25 C and 2400 ft-c. The incubation mixture contained 0.125 μmole of NH₄Cl, 0.30 μCi of purified ¹⁴C-glutamate, a range of monosodium glutamate concentrations, chloroplasts equivalent to 25.4 μg of Chl, and solution C to final volume of 0.125 ml. Percentage of intact plastids in the resuspension was determined to be 53.6%.

The standardized radiochemical assay was used to test the relative capability of intact chloroplasts to synthesize glutamine in the presence or absence of different nitrogen sources in the light and in darkness. Table I shows that very low but measurable rates of glutamine synthesis were obtained in darkness in the presence or absence of an exogenous nitrogen source. Incandescent illumination alone stimulated an 8-fold increase in synthesis relative to the dark control. When plastids were incubated with 10⁻³ M NH₄Cl, the rate of glutamine synthesis in light was enhanced 22.7 times relative to its dark level when NO₂⁻ was used as a nitrogen donor. Since light-driven glutamine synthesis is an intact plastid function (25), and since the plastids in this population were 60% intact, only the light-stimulated component of each reaction was corrected for 100% intact chloroplasts.

The light dependency of whole chloroplast glutamine synthesis and the stoichiometric requirement of glutamine synthetase for ATP suggest direct coupling of glutamine formation to photophosphorylation. By means of specific inhibitors of photosynthetic processes and controls of light quality, parallel correlations were tested between glutamine and ATP synthesis.

The controls of the experiment presented in Table II clearly indicate that far red as well as unfiltered incandescent illumination support glutamine synthesis by intact spinach chloroplasts. When plastids were incubated in the presence of 2 × 10⁻³ M salicylaldoxime, glutamine synthesis was inhibited significantly.
in far red but not in white light. DCMU at $2 \times 10^{-7}$ M, which was demonstrated to inhibit $O_2$ evolution 85% in similar preparations in white light, inhibited glutamine synthesis only 25%. In far red light there was no inhibition from DCMU. Finally, $10^{-6}$ M CCCP (13) completely inhibited glutamine synthesis in either white or far red light.

**DISCUSSION**

Reductive amidation as well as amination provide mechanisms by which NH$_4^+$ entering or produced in the chloroplast can be incorporated into organic compounds. The occurrence of such mechanisms are quite important in that they provide reduced nitrogen in a form available for further reactions. In addition, such reactions reduce the accumulation of free NH$_4^+$, which could otherwise uncouple photophosphorylation and promote regulatory effects on carbon metabolism (18).

In their initial observations of glutamine synthesis isolated chloroplasts, Santerius and Stocking (25) found no stimulation by exogenous NH$_4^+$, but additions were confined to no more than $10^{-4}$ M NH$_4$Cl. The data of Figure 2 from the present investigation confirm this observation, but also show that higher concentrations up to $10^{-3}$ M NH$_4^+$ stimulate whole plastid glutamine synthesis above endogenous nitrogen levels. At any given concentration of exogenous NH$_4^+$, the absolute level within the intact chloroplast is unknown, although it presumably increases with increasing external concentration. It is known however, that such high NH$_4^+$ concentrations as used would maximally uncouple photophosphorylation if incubated with class II chloroplasts without a limiting membrane (16). In the experiment of Figure 2, for example, the plastid preparation made a maximum of 88 nmoles of glutamine from 125 nmoles of exogenous glutamate. This amount of incorporation reduced the total available concentration of NH$_4^+$ from $10^{-4}$ M to 2.96 $\times 10^{-5}$ M, a concentration which, if surrounding the phosphoryrating membranes, could still uncouple significantly (16). Of course the outer envelope may restrict NH$_4^+$ uptake, but apparently it does so much less than glutamate uptake. Because the glutamate concentration in the standard assay is 50 times greater than the available NH$_4^+$, concentration, the possibility exists for eventual complete incorporation of free NH$_4^+$. However, beyond 12 min of reaction time, NH$_4^+$ incorporation by pea chloroplasts ceases before complete exhaustion of NH$_4^+$ takes place. This is in contrast to the situation with isolated spinach chloroplasts in which glutamine synthesis was observed at an ever decreasing rate for at least 30 min (25). It is possible that isolated whole pea chloroplasts are uncoupled more readily than those of spinach, so that glutamine synthesis stops after 12 min for lack of ATP. Because the isolation medium used was originally designed for maximum in vitro photosynthetic performance of spinach chloroplasts, it is also possible that the more rapid loss of activity by pea plastids reflects a lesser degree of suitability of the medium for pea.

It was concluded earlier that exogenous glutamate can penetrate rapidly into intact spinach chloroplasts (25). However, the outer envelope of pea chloroplasts seems to offer significant resistance to the free passage of added glutamate. It proved impossible to saturate intact plastid glutamine synthesis under conditions in which NH$_4^+$ and ATP presumably were not limited (Fig. 3). The view that the chloroplast envelope is only moderately permeable to glutamate is supported by the work of Heldt and Sauer (11) and Jacobson and Stumpf (15). Heldt and coworkers (12) have shown that glutamate penetrates the envelope of spinach chloroplasts via the dicarboxylate translocator at about 10 $\mu$moles mg Chl$^{-1}$ hr$^{-1}$ at 4 C from a medium containing 1 mm glutamate. In the present investigation, pea chloroplasts were found to synthesize glutamate at a rate of 26 $\mu$moles mg Chl$^{-1}$ hr$^{-1}$ (Fig. 2) at 25 C from a medium containing 50 mm glutamate, 2 mm NH$_4^+$, and ADP. It was also found that the concentration of glutamate in the medium could be increased without a limiting membrane (16), such that the plastids were maximally uncoupled if incubated with the absolute level of NH$_4^+$ concentration of 88 nmoles of glutamine from 125 nmoles of exogenous glutamate. This is in contrast to the situation with isolated pea chloroplasts where complete exhaustion of glutamate was noted before reaching the limiting membrane (16). This is in contrast to the situation with isolated pea chloroplasts where complete exhaustion of glutamate was noted before reaching the limiting membrane (16). The plastid preparation made a maximum of 88 nmoles of glutamine from 125 nmoles of exogenous glutamate. This amount of incorporation reduced the total available concentration of NH$_4^+$ from $10^{-4}$ M to 2.96 $\times 10^{-5}$ M, a concentration which, if surrounding the phosphoryrating membranes, could still uncouple significantly (16). Of course the outer envelope may restrict NH$_4^+$ uptake, but apparently it does so much less than glutamate uptake. Because the glutamate concentration in the standard assay is 50 times greater than the available NH$_4^+$ concentration, the possibility exists for eventual complete incorporation of free NH$_4^+$. However, beyond 12 min of reaction time, NH$_4^+$ incorporation by pea chloroplasts ceases before complete exhaustion of NH$_4^+$ takes place. This is in contrast to the situation with isolated spinach chloroplasts in which glutamine synthesis was observed at an ever decreasing rate for at least 30 min (25). It is possible that isolated whole pea chloroplasts are uncoupled more readily than those of spinach, so that glutamine synthesis stops after 12 min for lack of ATP. Because the isolation medium used was originally designed for maximum in vitro photosynthetic performance of spinach chloroplasts, it is also possible that the more rapid loss of activity by pea plastids reflects a lesser degree of suitability of the medium for pea.

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consistent with reports of permeability, enzyme compartmentation, and kinetics of intracellular movement (7, 10, 19, 23-25, 27-29). The quantitative significance of in vivo glutamine synthesis as a means of entrance of reduced nitrogen into organic combination and the regulatory roles of the enzyme glutamine synthetase in photosynthetic systems under different conditions of nitrogen form and availability are unknown and remain to be determined.

**LITERATURE CITED**


