Plant Physiol. (1994) 105: 415–424

A Metabolic Control Analysis of the Glutamine Synthetase/ Glutamate Synthase Cycle in Isolated Barley (*Hordeum vulgare* L.) Chloroplasts[1]

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Ammonia assimilation in chloroplasts occurs via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle. To determine the extent to which these enzymes contribute to the control of ammonia assimilation, a metabolic control analysis was performed on isolated barley (*Hordeum vulgare* L.) leaf chloroplasts. Pathway flux was measured polarographically as ammonium-plus-2-oxoglutarate-plus-glutamine-dependent O_2 evolution in illuminated chloroplasts. Enzyme activity was modulated by titration with specific, irreversible inhibitors of GS (phosphinothricin) and GOGAT (azaserine). Flux control coefficients ($C^{J^0}_{E_0}$) were determined (a) by differentiation of best-fit hyperbolic curves of the data sets (flux versus enzyme activity), and (b) from estimates of the deviation indices ($D^{J^r}_{E_i}$). Both analyses gave similar values for the coefficients. The control coefficient for GS was relatively high and the value did not change significantly with changes in 2-oxoglutarate concentration ($C^{J^0}_{E_0}$ = 0.58 at 5 mM 2-oxoglutarate and 0.40 at 20 mM 2-oxoglutarate). The control coefficient for GOGAT decreased with decreasing glutamine concentrations, from 0.76 at 20 mM glutamine to 0.19 at 10 mM glutamine. Thus, at high concentrations of glutamine, GOGAT exerts a major control over flux with a significant contribution also from GS. At lower concentrations of glutamine, however, GOGAT exerts far less control over pathway flux.

Ammonia assimilation in higher plants proceeds via GS and GOGAT, together termed the GS/GOGAT cycle. In barley (*Hordeum vulgare*), analysis of photorespiratory mutants indicates that this assimilation occurs almost exclusively in the chloroplast (Kendall et al., 1986; Wallsgrove et al., 1987). Although the process has been extensively studied (for recent reviews, see Sechley et al., 1992; Wallsgrove et al., 1992), its regulation is poorly understood. To determine the extent to which these two enzymes contribute to the control of ammonia assimilation, we have performed a metabolic control analysis of the pathway.

Metabolic control analysis, first introduced by Kacser and Burns (1973) and Heinrich and Rapoport (1974), provides a means of quantitatively determining the distribution of control throughout a metabolic pathway. Through this analysis it has become apparent that the control of a pathway is rarely, if ever, mediated through a single "rate-limiting" or "bottleneck" reaction. Rather, it is shared (albeit unequally) throughout the reactions of the pathway. The extent to which each enzyme contributes to control is determined by evaluating its flux control coefficient ($C^{J^0}_{E_0}$). One method of determining this value is to change the activity of a single enzyme within a pathway, for example, by titration with a specific inhibitor, and to measure the resulting change in flux through the pathway. The control coefficient is then calculated from the following equation:

$$C^{J^0}_{E^0} = \frac{dJ^0/J^0}{dE^0_i/E^0_i} \quad (1)$$

where E_i is an enzyme, E^0_i is the original concentration of the enzyme E_i, J^0 is the flux at the original enzyme concentration, and $C^{J^0}_{E_0}$ is the control coefficient for the enzyme E_i. In practical terms, the coefficient may be determined from titration curves of J (flux) against I (inhibitor concentration) and E (enzyme activity) against I. The control coefficient may then be calculated from the initial slopes of each titration curve,

$$C^{J^0}_{E^0} = \left(\frac{dJ}{J \cdot dI} \Big/ \frac{dE_i}{E_i \cdot dI}\right)^{I \to 0} \quad (2)$$

The analysis depends on measuring infinitesimally small changes in enzyme activity and flux, usually by extrapolation of the data to the initial, uninhibited flux (i.e. when $I = 0$). Very small changes in activity may, however, be difficult to measure with sufficient accuracy and reproducibility in biological systems. To take account of this, Small and Kacser (1993) introduced the deviation index ($D^{J^r}_{E_i}$), which gives a measure of the relative change in a metabolic variable (e.g.

[1] Financial support came from the Agricultural and Food Research Council (U.K.) grant No. PG34/543 and The Royal Society (University Research Fellowship to A.K.T.).

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Abbreviations: aza, azaserine; Fd_{ox}, oxidized ferredoxin; Fd_{red}, reduced ferredoxin; GOGAT, glutamate synthase; GS, glutamine synthetase; GS1, cytosolic glutamine synthetase; GS2, chloroplastic glutamine synthetase; PPT, phosphinothricin.

flux) due to a large (i.e. noninfinitesimal) relative change in a parameter (e.g enzyme concentration). This is defined as:

$$D^{J^r}_{E^r_i} = \left(\frac{\Delta J}{\Delta E_i}\right) \cdot \frac{E^r_i}{J^r} \quad (3)$$

where $\Delta E_i = E^r_i - E^0_i$, E^0_i is the original concentration of enzyme E_i, E^r_i is the new concentration of enzyme E_i (for example, following inhibition by I), $\Delta J = J^r - J^0$, J^0 is the flux at the original concentration of E_i, and J^r is the resulting flux after the enzyme activity has changed by a factor r. The deviation index between the points (E^r and J^r, E^0 and J^0) is equal to the flux control coefficient at the original enzyme concentration:

$$D^{J^r}_{E^r_i} \equiv C^{J^0}_{E^0_i} \quad (4)$$

In the present study both approaches (i.e. Eqs. 2 and 3) have been adopted to determine control coefficients for GS and GOGAT during ammonia assimilation in isolated chloroplasts.

The GS/GOGAT cycle of ammonia assimilation in chloroplasts is closely associated with the photosynthetic electron transport chain, which serves as the source of reductant (Fd_{red}) and ATP (Fig. 1). This relationship has been demonstrated in isolated pea (*Pisum sativum*) (Anderson and Done, 1977a, 1977b), spinach (*Spinacia oleracea*) (Woo, 1983), and barley (*Hordeum vulgare*) (Wallsgrove et al., 1986) chloroplasts, where the addition of GS/GOGAT-cycle substrates resulted in light-dependent O_2 evolution. In the present study the rate of ammonium-plus-2-oxoglutarate-plus-Gln-dependent O_2 evolution in illuminated barley chloroplasts has therefore been used as a measure of the flux (J) through the GS/GOGAT cycle. Control coefficients ($C^{J^0}_{E^0_i}$) have then been calculated from hyperbolic curve fits of J against E_i, where E_i is varied by titration with specific, irreversible inhibitors of GS (PPT) and GOGAT (aza). A comparison is made between these values and calculated deviation indices ($D^{J^r}_{E^r_i}$) at different concentrations of substrate. The extent to which GS and GOGAT contribute to the control of ammonia assimilation in isolated chloroplasts is discussed in relation to these coefficients.

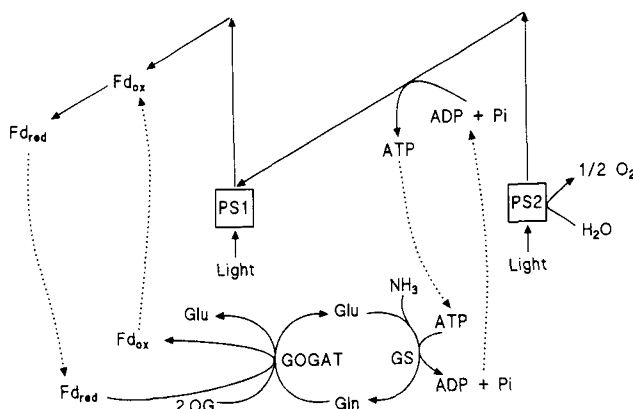

Figure 1. The relationship between the photosynthetic electron transport chain and the GS/GOGAT cycle.

MATERIALS AND METHODS

Growth of Plants

Barley (*Hordeum vulgare* L. cv Maris Mink) seeds were grown in Fisons Levington compost in a controlled environment cabinet (Sanyo Gallenkamp, UK) at 20°C (day)/10°C (night). The fluence rate was 350 μmol m^{-2} s^{-1} (PAR) over a 16-h photoperiod. RH was maintained at 65% and the plants were routinely harvested at least 1.5 h into the light period.

Protoplast Isolation

The procedure was modified from that of Wallsgrove et al. (1986). Primary leaves were excised from 8- to 10-d-old barley plants and the lower epidermis was removed. Leaves were placed with the stripped surface down into 50 mL of digestion medium (0.4 M sorbitol, 10 mM Mes, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1.5% [w/v] cellulase [Onozuka R10, Calbiochem, Welwyn, UK], pH 5.5) and incubated for 2 h in the light (illuminated under a 60-W tungsten lamp) at 29°C. Protoplasts were harvested by gentle mixing of the medium followed by filtration through a coarse nylon mesh into 50-mL centrifuge tubes. The protoplasts were pelleted by centrifugation at 250g for 5 min in a swing-out rotor at 4°C. The pellet was resuspended in 10 mL of 35% (v/v) Percoll in medium A (0.4 M sorbitol, 25 mM Tricine, 1 mM $CaCl_2$, 1 mM $MgSo_4$, pH 7.2). The suspension was divided between two 20-mL centrifuge tubes and overlayered with 2 mL of 25% (v/v) Percoll in medium A, followed by 1 mL of medium A. The gradients were left on ice for 1 h and then centrifuged at 300g for 5 min in a swing-out rotor at 4°C. Intact, mesophyll cell protoplasts were harvested from the interface between the 25% (v/v) Percoll and medium A layers.

Isolation of Chloroplasts from Protoplasts

Protoplasts were diluted approximately 4-fold with resuspension medium (0.33 M sorbitol, 50 mM Tricine, 2 mM EDTA, 1 mM $MgSO_4$, 1 mM $MnSO_4$, pH 7.8) and broken by passage through a 20-μm pore size nylon mesh. Chloroplasts were pelleted by centrifugation at 150g for 2 min using a swing-out rotor. The pellet was gently resuspended in 0.5 mL of assay medium (0.33 M sorbitol, 50 mM Tricine, 2 mM EDTA, 1 mM $MgSO_4$, 1 mM $MnSO_4$, pH 8.2). The entire procedure was carried out at 4°C.

Assay of CO_2-Dependent O_2 Evolution, Intactness, and Purity of Isolated Chloroplasts

Rates of CO_2-dependent O_2 evolution were measured using a Hansatech O_2 electrode (Hansatech, Kings Lynn, UK). Protoplasts or chloroplasts (50 μg Chl/assay) were incubated in the O_2 electrode at 25°C in 1 mL of assay medium (see above) containing 20 mM $KHCO_3$. The addition of 0.2 mM KH_2PO_4 was required for optimal rates of O_2 evolution by chloroplasts. Illumination was provided by a quartz halogen bulb (>1000 μmol m^{-2} s^{-1} PAR). Rates of O_2 evolution were routinely 100 μmol h^{-1} mg^{-1} Chl for protoplasts and 40 μmol h^{-1} mg^{-1} Chl for isolated chloroplasts. Chl concentration was determined according to the method of Arnon (1949).

The chloroplasts were routinely greater than 90% intact as determined by the ferricyanide assay (Lilley et al., 1975). Contamination of the chloroplasts by other cellular components was determined, by analysis of marker enzymes, to be <4% cytosolic (PEP carboxylase; Foster et al., 1982), 0.03% mitochondrial (citrate synthase; Cooper and Beevers, 1969), and <14% peroxisomal (glycolate oxidase; Behrends et al., 1982).

Measurement of Ammonium-plus-2-Oxoglutarate-plus-Gln-Dependent O_2 Evolution in Isolated Barley Chloroplasts

Rates of O_2 evolution were measured at 25°C in a Hansatech O_2 electrode (Hansatech, Kings Lynn, UK). Chloroplasts (100 μg of Chl) were preincubated in assay medium (see above) in the electrode for 3 min with illumination (>1000 μmol m^{-2} s^{-1} PAR) prior to the addition of the following substrates: Gln (10 or 20 mM), 2-oxoglutarate (5, 10, or 20 mM), and 0.5 mM $(NH_4)_2SO_4$. The final volume was 1.0 mL. Rates were measured at steady state 5 min after the addition of substrates, and the assay was illuminated throughout. Inhibitor titrations were performed by adding the inhibitor (PPT or aza) to the chloroplasts at the start of the preincubation period, i.e. 3 min before the addition of substrates.

Determination of Inhibition of GS and GOGAT in Situ following Inhibitor Titrations of Intact Chloroplasts

To determine the extent of enzyme inhibition following the addition of PPT or aza to the chloroplasts, as described in the previous section, the entire assay mixture was removed from the electrode at steady state (5 min after substrate addition) and divided into 100-μL aliquots. Each aliquot was layered onto a silicone oil gradient consisting of 20 μL of extraction buffer (either GS or GOGAT extraction buffer, as appropriate, with the addition of 20% [v/v] glycerol and omission of ethylene glycol) overlayered with 75 μL of silicone oil (AR200:AR20 in a ratio of 5:1 [v/v], Wacker Chemicals Ltd., Surrey, UK) in 400-μL polypropylene Eppendorf centrifuge tubes. The tubes were centrifuged at room temperature for 10 s at 12,680g in a horizontal rotor in a Beckman microfuge (Beckman UK, High Wycombe, UK).

Following centrifugation, the tubes were immediately frozen in liquid nitrogen and stored at −20°C prior to carrying out GS and GOGAT assays on the pelleted chloroplasts. This procedure separates intact chloroplasts from the rest of the assay mixture and removes inhibitor that has not entered the chloroplasts during the O_2 electrode assay. Because aza and PPT are irreversible inhibitors of barley leaf GOGAT and GS, respectively (A.C. Baron, A.K. Tobin, and R.M. Wallsgrove, unpublished data), subsequent determination of GS and GOGAT activity in the chloroplast pellet measures the extent of enzyme inhibition at steady state during the inhibitor titration experiments described above. Recovery of Chl from the original electrode assay was routinely greater than 90%.

Determination of GS Activity in the Chloroplast Pellet following Silicone Oil Centrifugation

The frozen tubes were cut through the silicone oil layer and pellets from each of 10 gradients were resuspended in 0.5 mL of GS extraction buffer (25 mM Tris, 1 mM EDTA, 1 mM DTT, 1 mM 2-mercaptoethanol, 1 mM reduced GSH, 10 mM $MgSO_4$, 5 mM glutamate, 2% [w/v] PVP, 10% [v/v] ethylene glycol, pH 8.0). GS activity was determined at 30°C using the transferase assay modified from Rhodes et al. (1975). The reaction mixture (final volume 100 μL) contained 80 mM Trizma base, pH 6.4, 64 mM Gln, 16 mM hydroxylamine, 2.24 mM $MnCl_2$, 0.24 mM ADP, and 25 mM sodium arsenate. The reaction was started with the addition of 10 μL of extract (2 μg Chl/assay) and stopped after 30 min with the addition of 100 μL of ferric chloride solution (0.24 M TCA, 0.1 M ferric chloride, 1.0 M HCl). Assays were carried out in microtiter plates and the A_{492} was measured using an SLT EAR 400 ELISA plate reader (SLT Laboratories, Vienna, Austria). The amount of product formed in the reaction was quantified by interpolation of a calibration curve using γ-glutamyl hydroxamate as the standard.

Determination of GOGAT Activity in the Chloroplast Pellet following Silicone Oil Centrifugation

The frozen tubes were cut through the silicone oil layer and the pellets from each of 10 gradients were resuspended in 0.5 mL of GOGAT extraction buffer (100 mM KH_2PO_4, 100 mM KCl, 5 mM EDTA, 0.1% [v/v] 2-mercaptoethanol, 0.1% [v/v] Triton X-100, 0.5 mM PMSF, pH 7.5). GOGAT activity was measured at 30°C using a modified assay from Wallsgrove et al. (1982). The assay mixture contained 100 mM KH_2PO_4, pH 7.5, 5 mM Gln, 5 mM 2-oxoglutarate, 1.2 mM methyl viologen, and 50 μL of chloroplast extract, in a final volume of 0.95 mL. The reaction was started with the addition of 50 μL of sodium dithionite (25 mg mL^{-1} in 0.3 M $NaHCO_3$). The reaction was stopped after 15 min by vortex mixing the assay tube until the methyl viologen had been completely oxidized, i.e. when the deep-blue color of reduced methyl viologen was no longer apparent. The assay mixture was then loaded onto a Dowex column (2 mL of Dowex 1 analytical grade [4% cross-linked, 200–400 mesh, prepared in the acetate form] in Bio-Rad prep plastic columns [Bio-Rad, Hertshire, UK]). The columns were washed with 20 mL of double-distilled water to remove Gln, and the bound glutamate was eluted by adding 5 mL of 2 N acetic acid. A 0.5-mL aliquot of this fraction was added to 1.0 mL of ninhydrin reagent (Matoh et al., 1980), and this was heated for 10 min at 80°C and cooled on ice for 10 min. The A_{506} was measured. Glutamate concentrations were calculated from a standardized calibration curve. Recoveries of glutamate and Gln from the Dowex columns were approximately 100%.

Inhibitor Titrations on the Isolated Enzymes

Partial Purification of Chloroplast Gln Synthetase from Barley Leaves

The extraction procedure was modified from that of Tobin et al. (1985). Barley primary leaves (8–10 d old) were frozen

and ground to a powder in liquid nitrogen in a pestle and mortar. GS extraction buffer (see above) was added at a concentration of 2.0 mL/g fresh weight of tissue. The homogenate was filtered through four layers of muslin and centrifuged at 14,270*g* for 30 min at 4°C in a Sorvall high-speed centrifuge (SS34 8 × 50 mL fixed-angle rotor). For each separate FPLC fractionation, a 3.0-mL aliquot of the supernatant was injected through a 0.22-μm mesh size 25-mm disc filter assembly (Millipore U.K. Ltd, Herts, UK). Two milliliters of the filtrate was injected onto a Mono Q anion-exchange column (HR5/5) attached to a FPLC system (Pharmacia LKB Biotechnology U.K., Milton Keynes, UK). The Mono Q column had been preequilibrated with 30 mL of elution buffer (25 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM DTT, 10% [v/v] ethylene glycol, pH 7.6). Fractions were eluted from the column using a combined stepped and linear gradient between 0 and 0.4 M NaCl at a flow rate of 1.0 mL min^{-1} (A.C. Baron, R.M. Wallsgrove, and A.K. Tobin, unpublished data). Fractions (1.0 mL) were collected and assayed for GS activity using the transferase assay. FPLC was performed at room temperature. Two peaks of GS activity eluted from the Mono Q column. The GS1 and GS2 isoforms eluted at 0.15 and 0.35 M NaCl, respectively.

The GS2 peaks from eight separate Mono Q fractionations were pooled and concentrated, first by dialysis against solid Suc for 2 h at 4°C, and then by the addition of dry Sephadex G25-300 (bead size 100-300 μm) at 50 mg mL^{-1} sample. After 20 min at 4°C the mixture was then centrifuged at 1000*g* in a swing-out rotor at 4°C and the supernatant was used as the partially purified GS2 preparation.

The GS2 preparation was free of contamination with GS1 as determined by SDS-PAGE of the preparation and western blotting with an antibody raised against the nodule-specific GS isoform from *Phaseolus* root nodules. This antibody recognizes both GS isoforms from leaves but did not detect any GS1 in the GS2 preparation (data not shown).

PPT Titration of Partially Purified GS2

PPT inhibition of GS2 was determined using the GS assay (Rhodes et al., 1975). The reaction mixture (0.1 mL final volume) contained 50 mM Tris-HCl, pH 7.8, 21 mM $MgSO_4$, 8 mM glutamate, 6 mM hydroxylamine, 4.5 mM EDTA, 8 mM ATP, and a range of PPT concentrations (0-50 μM). The reaction was started with the addition of GS2 and stopped after 30 min with the addition of 0.1 mL of ferric chloride solution (see above). Assays were carried out in microtiter plates and the absorbance was measured as described for the transferase assay.

aza Titration of a Crude GOGAT Preparation from Barley Leaves

Barley primary leaves (8-10 d old) were ground to a powder with liquid nitrogen in a pestle and mortar and extracted in GOGAT extraction buffer (see above). The homogenate was filtered through four layers of muslin and centrifuged at 14,270*g* for 30 min at 4°C in a Sorvall Superspeed centrifuge (SS34 8 × 50 mL fixed-angle rotor). Proteins were precipitated from the supernatant by fractionation with ammonium sulfate between 25 and 60% (w/v) saturation. Pellets were resuspended in 0.5 mL of GOGAT extraction buffer minus Triton X-100 and stored at −20°C until use. Thawed preparations were desalted on Sephadex G-25 columns (equilibrated with GOGAT extraction buffer minus Triton X-100 and PMSF) before assaying for GOGAT activity as described above. Inhibitor titrations were performed by adding aza (0-140 μM) to the reaction mixture prior to the addition of sodium dithionite to initiate the reaction.

Computer Curve Fitting

To calculate flux control coefficients from plots of J (rate of ammonium-plus-2-oxoglutarate-plus-Gln-dependent O_2 evolution [μmol O_2 h^{-1} mg^{-1} Chl]) against E_i, the data were curve-fitted to the equation:

$$J = \frac{A \cdot E_i}{B + E_i}, \quad (5)$$

a rectangular hyperbola. The curve-fit was performed using the Marquardt algorithm within the FigP software program (Biosoft, Cambridge, UK). Confidence limits were set at 95% with statistical weighting applied to each y value.

RESULTS

Although isolated barley chloroplasts evolved O_2 in the light in the presence of 2-oxoglutarate and ammonium alone, the rates were low (2-3 μmol O_2 h^{-1} mg^{-1} Chl) and nonlinear (Fig. 2). Therefore, it was necessary to include Gln as a substrate in addition to 2-oxoglutarate and ammonium to obtain optimal rates of O_2 evolution. This has been reported previously for barley chloroplasts isolated from protoplasts (Wallsgrove et al., 1986), whereas mechanically isolated pea chloroplasts do not require the addition of Gln (Anderson and Done, 1977a, 1977b). This difference may be due to a lower endogenous concentration of amino acids in chloroplasts isolated from protoplasts (A.C. Baron, R.M. Wallsgrove, and A.K. Tobin, unpublished data).

O_2 evolution was light dependent, did not occur when Gln, 2-oxoglutarate, or ammonium was added individually (Fig. 2), and was not detectable when the chloroplasts were broken osmotically (data not shown). The addition of either 10 mM glyceraldehyde, which completely inhibited CO_2-dependent O_2 evolution, or of catalase to remove any H_2O_2 present did not significantly affect the rate of ammonium-plus-2-oxoglutarate-plus-Gln-dependent O_2 evolution (data not shown). This suggests that there was no interference from either CO_2-dependent O_2 evolution or of the Mehler reaction on the measurements of GS/GOGAT cycle flux.

Control at the GS Reaction

PPT was used to alter the activity of GS in isolated chloroplasts. There was no effect of PPT on CO_2-dependent O_2 evolution by isolated chloroplasts at the concentrations used in this study, nor was there any effect of PPT on GOGAT activity (data not shown). PPT was found to be an irreversible inhibitor of GS from barley leaves and ATP was essential for irreversible binding to occur (A.C. Baron, A.K. Tobin, and

R.M. Wallsgrove, unpublished data). Thus, it was necessary to preincubate chloroplasts in the light with PPT prior to the addition of substrates to ensure that sufficient ATP was generated, via photophosphorylation, to enable the inhibitor to bind to the enzyme.

The response of the partially purified enzyme to PPT titration differed somewhat from that of the enzyme inside intact chloroplasts (Fig. 3). At a given concentration of PPT, the enzyme in vitro is always inhibited to a greater extent than it is within intact chloroplasts (in situ). One factor is that the stromal concentration of PPT at steady state is 25 to 35% less than that added to the chloroplasts (calculated from silicone oil centrifugation of [^{14}C]PPT-labeled chloroplasts; data not shown). This does not account entirely for the different responses, however, and a significant factor is likely to be the conditions to which the enzyme is exposed during PPT treatment, e.g. ATP, substrate concentration, and pH. The isolated enzyme is titrated with PPT in the presence of saturating ATP and substrate, and this may not accurately reflect the conditions existing within the chloroplast stroma. For this reason, and because we have already confirmed that PPT inhibition is irreversible, it was decided that the in situ titration curves provided a more accurate estimate of GS inhibition at steady-state flux, so this technique was employed in all subsequent analyses.

Figure 4, A–C, shows plots of the steady-state rate of ammonium-plus-2-oxoglutarate-plus-Gln-dependent O_2 evolution (J) against GS activity (E_i) in isolated barley chloro-

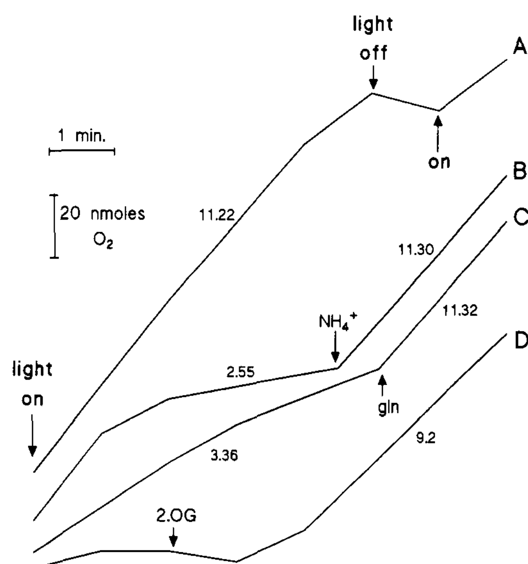

Figure 2. O_2 evolution in intact barley chloroplasts. Numbers on traces refer to the rate of O_2 evolution (μmol O_2 h^{-1} mg^{-1} Chl). Chloroplasts (100 μg Chl/assay) were incubated in an O_2 electrode as described in "Materials and Methods." The following additions were made: A, 20 mM Gln, 20 mM 2-oxoglutarate, 0.5 mM $(NH_4)_2SO_4$ (at $t = 0$); B, 20 mM 2-oxoglutarate, 20 mM Gln (at $t = 0$), 0.5 mM $(NH_4)_2SO_4$ was added where shown; C, 20 mM 2-oxoglutarate, 0.5 mM $(NH_4)_2SO_4$ (at $t = 0$), 20 mM Gln was added where shown; D, 20 mM Gln, 0.5 mM $(NH_4)_2SO_4$ (at $t = 0$), 20 mM 2-oxoglutarate was added where shown.

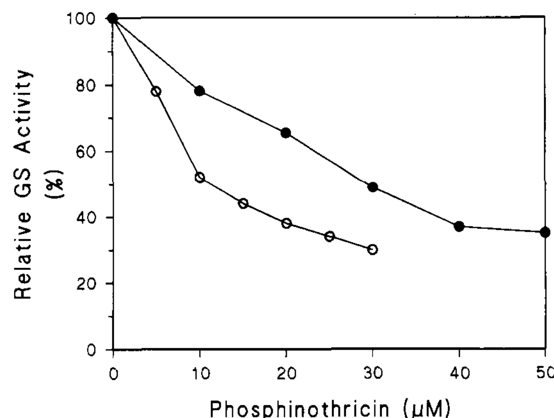

Figure 3. Effect of PPT on GS activity in partially purified GS (GS2) from barley chloroplasts (in vitro) (O) and intact chloroplasts (in situ) (●). For the in situ titrations, chloroplasts were preincubated in the light with PPT prior to the addition of substrates [0.5 mM $(NH_4^+)_2SO_4$, 20 mM 2-oxoglutarate, and 20 mM Gln]. Chloroplasts were then sampled at steady state, centrifuged through a silicone oil layer, and assayed for remaining GS activity. Further experimental details are given in "Materials and Methods." Control (100%) rates were 414.0 nKatals mg^{-1} Chl (●, in situ, GS transferase assay) and 33.0 nKatals mg^{-1} protein (O, in vitro, GS assay). Each point represents the mean of three separate determinations.

plasts at three different concentrations of 2-oxoglutarate. GS activity was varied by titration with PPT. To calculate $C^{J^0}_{E_i^0}$, as given in Equation 2, it is necessary to determine the gradient of the tangent to the curve at (J^0, E^0). This was achieved mathematically by differentiation of the best-fit equation of the curve for each data set, as follows:

In each case the data have been curve-fitted to a rectangular hyperbola with the equation:

$$J = \frac{A \cdot E_i}{(B + E_i)} \quad (6)$$

where A and B are independent variables. Differentiation of this equation, with respect to E, gives:

$$\frac{dJ}{dE_i} = \frac{AB}{(B + E_i)^2} \quad (7)$$

Multiplication by E_i^0/J^0 gives the value of the initial slope through the point (E^0, J^0), i.e. the flux control coefficient:

$$C^{J^0}_{E^0} = \left(\frac{\delta J}{\delta E_i}\right) \cdot \frac{E_i^0}{J^0} = \frac{AB}{(B + E_i^0)^2} \cdot \frac{E^0}{J^0} \quad (8)$$

Thus, substitution of values for E_i^0, J^0, A, and B (derived from best-fit parameters of the curve-fit to Equation 6, see "Materials and Methods") into Equation 8 gives the control coefficient at the initial, uninhibited flux. The values of J^0 were derived by extrapolation of the curve-fit of Equation 6 through E^0. The control coefficients are shown in Table I.

In all cases, the data fit to a rectangular hyperbola (Fig. 4), as predicted for linear, unbranched pathways (Small and Kacser, 1993). It is thus valid to calculate deviation indices ($D^{J^r}_{E_i}$) between J^0 and each value of J^r, as given in Equation 3.

According to Small and Kacser (1993), providing that the data fit to a rectangular hyperbola, the value for $D^{J^r}_{E_i}$ should be the same for all values of J^r. The mean value of $D^{J^r}_{E_i}$ for each of the data sets in Figure 4 are shown in Table I. The values of $D^{J^r}_{E_i}$ are quite comparable within each set of conditions, as indicated by the relatively small SE values. This is as expected given the hyperbolic fit of the curves (Small and Kacser, 1993). Mean values of $D^{J^r}_{E_i}$ in each case are in relatively good agreement with the control coefficients calculated from the differentiated curve fits.

At all concentrations of substrate, GS has a relatively high control coefficient, indicating that the enzyme has significant control of flux through the pathway (Table I). As the concentration of 2-oxoglutarate is increased from 5 to 20 mM, there is only a small, and probably insignificant, decrease in the values of $C^{J^0}_{E^0_i}$ from 0.58 to 0.40; and $D^{J^r}_{E_i}$ from 0.35 to 0.30. Thus, it would seem that although GS exerts some control over flux, this control is not affected by relatively large changes in concentration of 2-oxoglutarate.

Control at the GOGAT Reaction

aza titrations were performed on partially purified GOGAT and on chloroplasts carrying out ammonium-plus-2-oxoglutarate-plus-Gln-dependent O_2 evolution at two different concentrations of Gln. Inhibition of GOGAT in situ was determined as previously described for GS. As with PPT, there was no effect of aza on CO_2-dependent O_2 evolution in isolated chloroplasts at the concentrations used in this study, nor was there any effect of aza on GS activity (data not shown). aza was determined to be an irreversible inhibitor of barley leaf GOGAT. Inhibition was competitive with Gln and noncompetitive with 2-oxoglutarate (A.C. Baron, A.K. Tobin, and R.M. Wallsgrove, unpublished data). At a given concentration of aza, the isolated enzyme was inhibited more than the enzyme in situ (Fig. 5). By the same reasoning as that presented above for GS inhibition, it was decided that the in situ titration curve provided the more accurate representation of GOGAT inhibition within the chloroplasts, and these data were used in all subsequent analyses.

Table I. *Flux control coefficients ($C^{J^0}_{E^0_i}$) and deviation indices ($D^{J^r}_{E_i}$) for GS and GOGAT during ammonium-plus-2-oxoglutarate-plus-Gln-dependent O_2 evolution in illuminated barley leaf chloroplasts*

Control coefficients and deviation indices were calculated from the data in Figures 3 and 5, as described in "Materials and Methods." Values for the deviation indices are the means of all calculated values of ($D^{J^r}_{E_i}$) at each value of r (where r is the factor by which the enzyme concentration has been altered). SE values are shown in parentheses.

Enzyme	Substrate Concentrations	$C^{J^0}_{E^0 i}$	$D^{J^r}_{E_i}$
GS	20 mM 2-oxoglutarate	0.40	0.30
	20 mM Gln		(±0.03)
	10 mM 2-oxoglutarate	0.43	0.43
	20 mM Gln		(±0.08)
	5 mM 2-oxoglutarate	0.58	0.35
	20 mM Gln		(±0.05)
GOGAT	20 mM 2-oxoglutarate	0.76	0.64
	20 mM Gln		(±0.08)
	20 mM 2-oxoglutarate	0.19	0.19
	10 mM Gln		(±0.09)

Figure 6, A and B, are plots of the steady-state rate of ammonium-plus-2-oxoglutarate-plus-Gln-dependent O_2 evolution (J) against GOGAT activity (E_i) in isolated barley chloroplasts at two different concentrations of Gln. GOGAT activity was varied by titration with aza. The data fit to a rectangular hyperbola with relatively good correlation coefficients for the fit (see legend to Fig. 5).

Flux control coefficients for GOGAT were calculated from the differentiated curve fits of Figure 5 in the same way as for GS. These coefficients and values for $D^{J^r}_{E_i}$ are shown in Table I. Again, there is relatively good agreement between the $D^{J^r}_{E_i}$ values, the mean of these values, and the flux control coefficients. At high concentrations of Gln (20 mM) the coefficients for GOGAT are high ($C^{J^0}_{E^0_i}$ = 0.76, $D^{J^r}_{E_i}$ = 0.51), indicating that GOGAT exerts a major control over flux. Indeed, since the flux summation theorem (Kacser and Burns, 1973) predicts that the sum of all coefficients of the enzymes in a

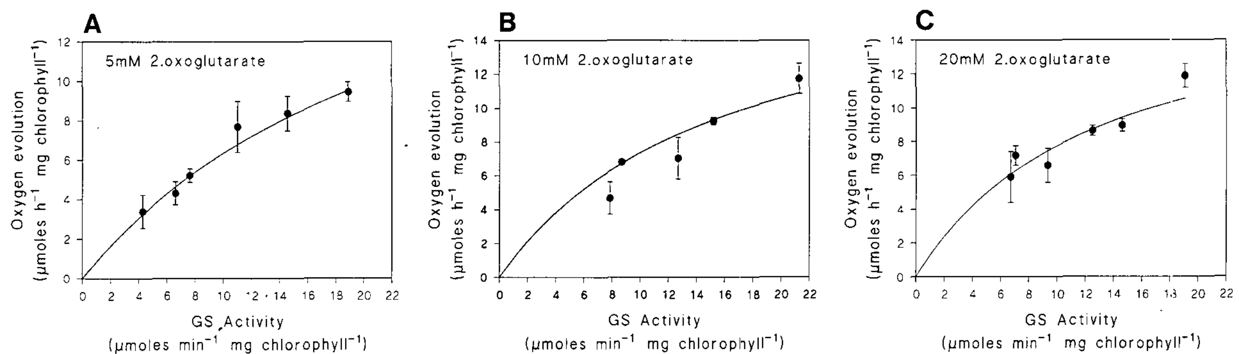

Figure 4. O_2 flux versus GS2 activity in isolated barley chloroplasts. Chloroplasts were preincubated in the light with PPT (0–50 μM) prior to the addition of 0.5 mM $(NH_4^+)_2SO_4$, 20 mM Gln, and 5 mM 2-oxoglutarate (A), 10 mM 2-oxoglutarate (B), or 20 mM 2-oxoglutarate (C). Rates were measured at steady state (5 min after the addition of substrate). GS activity was determined using the in situ sampling and assay technique. Each point represents the mean of five separate determinations on different chloroplast preparations (±SE). The data were curve-fitted to a rectangular hyperbola as described in "Materials and Methods." Correlation coefficients for each curve fit were 0.968, A; 0.822, B; 0.731, C.

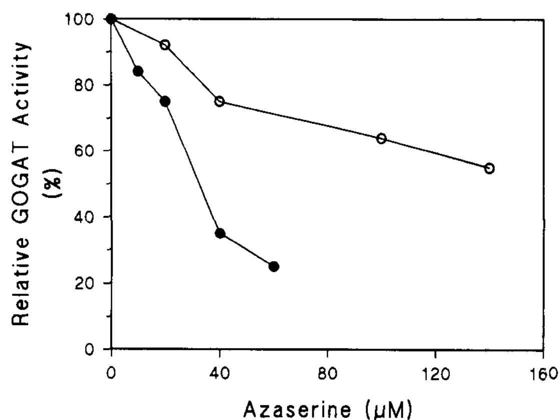

Figure 5. Effect of aza on GOGAT activity in partially purified GOGAT from barley chloroplasts (in vitro) (●) and intact chloroplasts (in situ) (○). For the in situ titrations, chloroplasts were preincubated in the light with aza prior to the addition of substrates [0.5 mM $(NH_4^+)_2SO_4$, 20 mM 2-oxoglutarate, and 20 mM Gln]. Chloroplasts were then sampled at steady state, centrifuged through a silicone oil layer, and assayed for remaining GOGAT activity. Further experimental details are given in "Materials and Methods." Control (100%) rates were 0.35 nKatals mg^{-1} protein (●, in vitro) and 3.0 nKatals mg^{-1} Chl (○, in situ). Each point represents the mean of three separate determinations.

pathway equals 1.0 (see, however, "Discussion"), this suggests that GOGAT exerts more control than all of the other steps in the pathway. As the Gln concentration is decreased to 10 mM, the control coefficients ($C^{J^0}_{E_i}$ and $D^{\Gamma}_{E_i}$ = 0.19) decrease considerably, indicating a major transfer of control away from GOGAT and toward other enzymes in the pathway. Thus, at high concentrations of Gln it would appear that pathway flux is predominantly controlled by GOGAT, with a significant contribution by GS ($C^{J^0}_{E_i}$ = 0.40), but as Gln concentrations fall, GOGAT contributes far less toward the overall control of ammonia assimilation in isolated chloroplasts.

DISCUSSION

The technique of metabolic control analysis has been applied to determine the extent to which GS and GOGAT contribute to the control of ammonia assimilation in isolated chloroplasts. In this study, ammonia assimilation was measured by determining the rate of O_2 evolution in the presence of GS/GOGAT cycle substrates ammonia, 2-oxoglutarate, and Gln. Although this is an indirect measurement of ammonia assimilation, previous studies with isolated pea (Anderson and Done, 1977a, 1977b), spinach (Woo, 1983), and barley (Wallsgrove et al., 1986) chloroplasts, in addition to the present study, indicate that the rate of O_2 evolution is directly related to the flux through the GS/GOGAT cycle. O_2 evolution is dependent on light and substrate addition, and requires intact chloroplasts (this study). Rates are inhibited by titration with GS inhibitors, L-Met sulfoximine (Anderson and Done, 1977a, 1977b) and PPT (this study), GOGAT inhibitors, aza (Anderson and Done, 1977a, 1977b; this study), and electron transport inhibitors, DCMU (Anderson and Done, 1977a, 1977). In pea chloroplasts it was shown that the amount of O_2 evolved in the presence of Gln and 2-oxoglutarate followed the predicted stoichiometry (Anderson and Done, 1977a, 1977b). Thus, it would seem that measurement of O_2 evolution is an appropriate means of determining GS/GOGAT cycle flux.

An alternative approach, which is currently being evaluated in our laboratory, is to determine the net rate of glutamate formation. This may be considered to be a more direct evaluation of GS/GOGAT cycle flux, but it is important to ascertain whether there is any further metabolism of glutamate within the chloroplast. It remains to be seen if the control coefficients are the same when these two different methods are used to measure flux (M. Ashraf, A.K. Tobin, and M.J. Emes, unpublished data).

Inhibitor titrations, using specific irreversible inhibitors of GS and GOGAT, were used to manipulate enzyme activities within the intact chloroplasts. Large differences were observed in the extent of inhibition of both enzymes depending on whether the inhibitor was added to the isolated enzyme

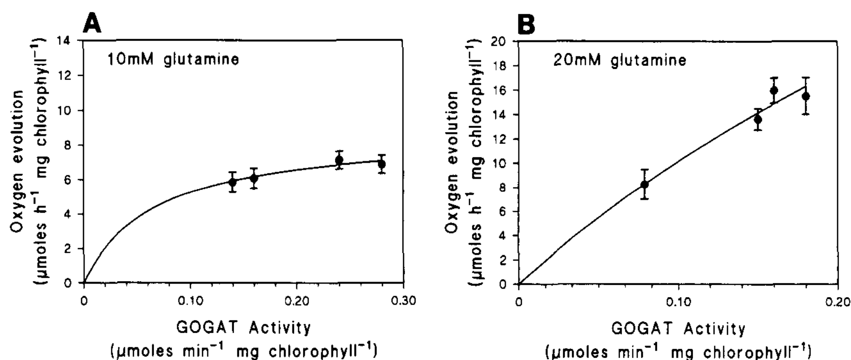

Figure 6. O_2 flux versus GOGAT activity in isolated barley chloroplasts. Chloroplasts were preincubated in the light with aza (0–140 μM) prior to the addition of 0.5 mM $(NH_4^+)_2SO_4$, 20 mM 2-oxoglutarate, and either 10 (A) or 20 mM Gln (B). Rates were measured at steady state (5 min after the addition of substrate). GOGAT activity was determined using the in situ sampling and assay technique. Each point represents the mean of five separate determinations on different chloroplast preparations (±SE). The data were curve fitted to a rectangular hyperbola as described in "Materials and Methods." Correlation coefficients for each curve fit were 0.926, A; 0.614, B.

or to the intact chloroplasts (in situ titration). In both cases at a given concentration of inhibitor, the enzyme was inhibited to a greater extent when it was titrated in vitro (i.e. as a partially purified preparation) than when it was treated within the intact chloroplasts. Because the latter condition was thought to give a more accurate estimate of enzyme activity at steady-state flux, this measurement was used in the analyses. It is essential to use irreversible inhibitors in this type of analysis because the formation of the enzyme-inhibitor complex must withstand the silicone oil fractionation and resuspension steps. aza and PPT were found to be specific, irreversible inhibitors of barley leaf GOGAT and GS, respectively (A.C. Baron, R.M. Wallsgrove, and A.K. Tobin, unpublished data); hence, they were considered to be appropriate inhibitors for in situ titration.

Two approaches were taken to determine flux control coefficients. In the first case, a best fit was made of Equation 7 for a rectangular hyperbola. Differentiation and scaling of the curve-fitted equation then allowed calculation of the flux control coefficients (Eq. 8). This type of analysis is dependent on having a hyperbolic relationship between flux and enzyme activity, as predicted for a linear pathway without branch points (see Small and Kacser, 1993). In all cases, the curves fit relatively closely to the data (Figs. 4 and 6; correlation coefficients range from 0.61–0.97), which indicates that the analysis is appropriate for the present study. The second approach was to calculate the deviation index ($D^{J^r}_{E_i}$) between J^0 and each value of J^r as given in Equation 3. The mean of all values of $D^{J^r}_{E_i}$ then provides an estimate of the flux control coefficient at (E^0, J^0). This technique is useful where relatively large changes occur in enzyme activity, e.g. when mutants or transgenic organisms are used (see Small and Kacser, 1993, for discussion). In the present study, it was found that the mean value of $D^{J^r}_{E_i}$ was approximately equal to the control coefficient estimated from curve fits of the data (Table I), thus supporting the validity of this analysis.

The data presented in Figures 4 and 6 might suggest that, in absolute terms, there is a 60-fold excess of GS activity, whereas there is insufficient GOGAT activity to support the measured fluxes, given that 4 mol of glutamate will be formed for each mol of O_2 evolved. However, it must be emphasized that it is the response, both of the enzymes and of the pathway, that is important in the analysis, and not the absolute activities. The response of GS to the inhibitor PPT was determined by measuring the transferase activity of the enzyme following silicone oil centrifugation of the chloroplasts (see "Materials and Methods"). Although the transferase assay is nonphysiological, it was necessary to use this more sensitive assay to accurately measure small changes in GS activity.

In addition, because ATP is required for binding of PPT to GS, and because the synthetase assay requires the addition of ATP, it was decided that this might lead to an overestimate of the extent of PPT inhibition, since any unbound PPT that was carried over during silicone oil centrifugation might become bound to the enzyme during the synthetase assay. Transferase activity was approximately 20 times higher than the synthetase activity in the chloroplast samples. Therefore, the measured rates of GS activity are overestimates of the GS capacity within the chloroplast. GOGAT activity is likely to be underestimated because the enzyme was extremely unstable following isolation (A.C. Baron, R.M. Wallsgrove, and A.K. Tobin, unpublished data). These points highlight the inadequacies of using in vitro measurements of enzyme activity to attempt to understand the in vivo control of pathway flux. The complex regulation under which an enzyme may operate in vivo, together with its mutual interdependence on other enzymes within the pathway, mean that in vitro measurements can only provide a simplified and probably incorrect understanding of flux control. Metabolic control analysis has the advantage of providing a quantitative means of analyzing the response of the pathway under in vivo conditions.

The present analysis has determined control coefficients only for GS and GOGAT, but there are other reactions involved in the pathway of ammonia assimilation in chloroplasts: metabolite translocators and the photosynthetic electron transport chain. Metabolite translocators are responsible for the import of the substrates 2-oxoglutarate and Gln into the chloroplast and for the export of glutamate into the medium. The influx of ammonia into the chloroplast has not been studied in detail in higher plants, although it is generally accepted that free ammonia (NH_3) diffuses across the envelope and into the stroma without the involvement of a membrane translocator (Kleiner, 1981). 2-Oxoglutarate is transported into the chloroplast via a dicarboxylate translocator, which counter-exchanges 1:1 with stromal dicarboxylates (Dry and Wiskich, 1983; Somerville and Ogren, 1983; Wallsgrove et al., 1986; Woo et al., 1987). There is evidence that more than one translocator is involved in 2-oxoglutarate uptake. Mutants of *Arabidopsis* (Somerville and Ogren, 1983) and barley (Wallsgrove et al., 1986), which lack the dicarboxylate translocator, have a reduced capacity to synthesize glutamate and also accumulate 2-oxoglutarate under photorespiratory conditions (Wallsgrove et al., 1986). The mutant plants grow and reproduce normally under conditions that prevent photorespiration but permit primary nitrogen assimilation (i.e. grown at 1% [v/v] CO_2). This suggests either that the rate of diffusion of 2-oxoglutarate across the chloroplast envelope is sufficient to support primary ammonia assimilation, which has been estimated to be approximately 10% of the rate of photorespiratory ammonia assimilation (Keys et al., 1978), or that there is more than one translocator involved in importing 2-oxoglutarate into the chloroplast (Dry and Wiskich, 1983; Woo et al., 1987). Gln transport into isolated chloroplasts is mediated by a Gln:glutamate translocator that may operate in either direction (Yu and Woo, 1988). Glutamate may also be exported by the glutamate:malate translocator (Yu and Woo, 1988). This latter reaction leads to a glutamate counter-exchange with malate, which can then exchange with 2-oxoglutarate via the dicarboxylate translocator (Dry and Wiskich, 1983).

As yet there are no specific inhibitors of any of these metabolite translocators; hence, it is not possible to undertake the same type of analysis performed here for GS and GOGAT. This leaves two alternative methods of determining the control coefficients for metabolite transport: (a) to determine control coefficients for all of the other reactions of the pathway, and, since the flux summation principle predicts that the sum of all coefficients of a pathway is 1.0, the remaining

control will be due to the translocators; or (b) use mutants or transgenic plants with altered chloroplast translocator activities.

Although the first method has been used in an analysis of the control of respiration in isolated plant mitochondria (Padovan et al., 1989), there are two inherent problems in employing this method. First, it would fail to distinguish between the different types of translocator. Second, it is likely that the sum of control coefficients in this pathway will be greater than 1.0 because the pathway involves a series of group-transfer reactions, i.e. the photosynthetic electron transport chain. It has recently been shown that where group-transfer reactions are involved in a pathway, the sum of flux control coefficients will be between 1.0 and 2.0. The actual sum depends on the extent of involvement of group-transfer reactions in the pathway (Van Dam et al., 1993).

In the present study, control coefficients were not estimated for the photosynthetic electron transport chain, and without this information it is not possible to make predictions about the likely sum of control coefficients of the pathway. Specific inhibitors are available for titration of the photosynthetic electron transport chain, and these analyses are currently being undertaken in our laboratory (M. Ashraf, A.K. Tobin, and M.J. Emes, unpublished data). The second approach, using mutant or transformed plants, will be valuable as soon as suitable plants become available. At present only one barley mutant has been isolated that appears to lack the ability to transport 2-oxoglutarate (Wallsgrove et al., 1986). It might be possible, through back-crossing with the wild type, to generate plants with intermediate levels of chloroplast 2-oxoglutarate transport activity, and these could be used in future analyses. The generation of transgenic lines with altered translocator activities, e.g. by antisense, awaits the isolation of cDNA clones for the individual translocators. This has yet to be achieved. Mutant and transgenic plants, however, have already been used successfully for metabolic control analysis of carbon assimilation pathways (Kruckeberg et al., 1989; Lauerer et al., 1993).

Although metabolic control analysis has yet to be applied to the ammonia assimilation pathway in whole plants, previous analyses of photorespiratory mutants of barley have been interpreted as indicating that GS and GOGAT are both "in excess." Mutants with altered chloroplast GS activities of between 8 and 160% of the wild type were transferred from high concentrations of CO_2 into normal air. Only plants with less than 38% of wild-type GS2 activity accumulated NH_4^+ in their leaves, and this was taken to indicate that there was a 2-fold excess of GS2 in barley leaves (Blackwell et al., 1987). The presence of the cytosolic GS isoenzyme may, however, lead to an underestimate of the control by GS2, since this offers an alternative route for ammonia assimilation in the mutants. Similar studies have yet to be performed with GOGAT mutants, although barley plants that completely lack Fd-GOGAT will grow and reproduce providing that the CO_2 concentration is high enough to prevent photorespiration. Therefore, it would seem that although there is at least 10-fold less NADH-dependent than Fd-dependent GOGAT activity in barley, there is adequate NADH-GOGAT activity in these plants to assimilate ammonia generated via primary nitrogen assimilation (Wallsgrove et al., 1982; Kendall et al., 1986).

There is evidence of a differential distribution of GOGAT isoenzymes in plant tissue. NADH-GOGAT predominates in nonphotosynthetic tissue, whereas Fd-GOGAT is the dominant isoenzyme in photosynthetic tissue (Yamaya et al., 1993). In the present study, we used chloroplasts that were isolated from mesophyll cell protoplasts and so the amount of NADH-GOGAT present is extremely low (L.C. Peat, T. Yamaya, and A.K. Tobin, unpublished data). This heterogeneity in distribution of the isoenzymes indicates that there are distinct roles for NADH- and Fd-GOGAT in leaf cells, a factor that must be considered when performing control analysis on whole tissue.

It is important to note that the value of a control coefficient depends on the experimental conditions under which it is evaluated. Control coefficients determined in in vitro systems may not necessarily reflect the situation in vivo, unless the in vivo conditions are accurately reproduced in vitro. GOGAT, for example, has a high control coefficient at high (20 mM) Gln concentrations, but the coefficient decreases considerably with a decrease in Gln concentration. One explanation for this response is that at high Gln concentrations GOGAT activity may be sufficient to account almost entirely for the rate of O_2 evolution, so that the contribution from GS becomes minimal. However, even at 20 mM Gln the control coefficient for GS is relatively high (0.4–0.53), so it would appear that the situation never arose where the rate of O_2 evolution became entirely dependent on GOGAT activity.

It is also important to note that the cytosolic Gln concentration is likely to be relatively low in vivo because the chloroplast is the major site of Gln formation in barley (Kendall et al., 1986; Wallsgrove et al., 1987). Therefore, it is unwise to make direct extrapolations from an analysis of isolated organelles in order to assign control coefficients to enzymes in vivo.

Nevertheless, the present study provides an opportunity to determine the way in which control is distributed between GS and GOGAT and to observe how this distribution changes with changes in substrate supply. Two significant changes were noted. First, the control coefficient for GS does not change with changes in the 2-oxoglutarate concentration. Second, the control coefficient for GOGAT is markedly decreased with decreasing Gln. This would tend to suggest that under photorespiratory conditions, assuming that the cytosolic Gln concentration is low, one might expect GOGAT to become less important in controlling flux. It is possible, although we have no direct evidence for this, that at low Gln concentrations the Gln translocator becomes a significant factor in the control of flux. Further analyses, under a wider range of substrate concentrations, are needed to test these hypotheses.

CONCLUSIONS

The results of this study provide the first quantitative analysis of control of ammonia assimilation in isolated chloroplasts. Two important conclusions are reached: the control of ammonia assimilation is shared between the enzymes in the pathway, and the distribution of control changes with

changes in substrate concentration. Without a detailed knowledge of the conditions present within the mesophyll cell chloroplasts in vivo, it is unwise to use the coefficients from this analysis to make predictions about the control of ammonia assimilation in the whole leaf. Nevertheless, this study provides an early and important insight into the control of ammonia assimilation in isolated chloroplasts.

ACKNOWLEDGMENT

We thank Dr. Brian Forde (Rothamsted) for the gift of the *Phaseolus* GS antibody.

Received October 13, 1993; accepted February 7, 1994.
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