Ammonia Assimilation by *Aspergillus nidulans*: [^{15}N]Ammonia Study

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^{15}N kinetic labelling studies were done on liquid cultures of wild-type *Aspergillus nidulans*. The labelling pattern of major amino acids under 'steady state' conditions suggests that glutamate and glutamine-amide are the early products of ammonia assimilation in *A. nidulans*. In the presence of phosphinothricin, an inhibitor of glutamine synthetase, ^{15}N labelling of glutamate, alanine and aspartate was maintained whereas the labelling of glutamine was low. This pattern of labelling is consistent with ammonia assimilation into glutamate via the glutamate dehydrogenase pathway. In the presence of azaserine, an inhibitor of glutamine synthase, glutamate was initially more highly labelled than any other amino acid, whereas its concentration declined. Isotope also accumulated in glutamine. Observations with these two inhibitors suggest that ammonia assimilation can occur concurrently via the glutamine synthetase/glutamate synthase and the glutamate dehydrogenase pathways in low-ammonia-grown *A. nidulans*. From a simple model it was estimated that about half of the glutamate was synthesized via the glutamate dehydrogenase pathway; the other half was formed from glutamine via the glutamate synthase pathway. The transfer coefficients of nine other amino acids were also determined.

INTRODUCTION

Two major routes exist in micro-organisms for the incorporation of ammonia into organic compounds: the amination reaction catalysed by NAD(P)H-GDH with the synthesis of glutamate (GDH pathway) and the amination of glutamate with the formation of glutamine (GS pathway). An alternative pathway of glutamate synthesis involves the reductive transfer of the amide nitrogen of glutamine to the 2-position of 2-oxoglutarate by GOGAT (GOGAT pathway; Tempest *et al.*, 1970). By coupling the GOGAT reaction with GS, an essentially irreversible pathway for the formation of glutamate is achieved. In some organisms, the combined GS/GOGAT pathways seem to be the only mechanism of ammonia assimilation for the synthesis of glutamate, when ammonia availability is limited (Hummelt & Mora, 1980a; Miflin & Lea, 1980; Tempest *et al.*, 1970).

However, GOGAT has only been detected in a few species of filamentous fungi and yeasts (Brown *et al.*, 1973; Casper *et al.*, 1985; Dunn-Coleman *et al.*, 1981; Hummelt & Mora, 1980b; Roon *et al.*, 1974). Furthermore, the role of GOGAT in glutamate synthesis is uncertain in some organisms due to the presence of high NAD(P)-GDH activities (Roon *et al.*, 1974).

In *Aspergillus nidulans* GOGAT has been detected and its participation in glutamate synthesis together with NADP-GDH suggested (Kusnan *et al.*, 1987). We present here further evidence from [^{15}N]ammonia studies for the concurrent operation of the NADP-GDH and GS/GOGAT pathways of ammonia assimilation in this organism.

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Abbreviations: γ -ABA, γ -aminobutyric acid; AZS, azaserine; GDH, glutamate dehydrogenase; GS, glutamine synthetase; GOGAT, glutamate synthase; MSO, methionine sulfoximine; PPT, phosphinothricin.

In order to evaluate the relative contribution of the GDH and GS/GOGAT pathways of ammonia assimilation to the formation of glutamate in *A. nidulans*, a model of ^{15}N labelling has been developed. Therefore, *A. nidulans* was grown on low ammonia and a quantitative analysis of the kinetics of ^{15}N incorporation into tissue amino acid pools was used. A similar approach has been successfully applied to the culture of *Candida utilis*, *Lemna minor* and *Hordeum vulgare* (Fentem *et al.*, 1983; Rhodes *et al.*, 1980; Sims & Folkes, 1964).

METHODS

Organism. *A. nidulans* wild-type (DSM 63358) obtained from Deutsche Sammlung für Mikroorganismen (Braunschweig, FRG) was grown in culture medium as described previously (Kusnan *et al.*, 1987) with 2 mM- NH_4Cl as sole nitrogen source. As a modification 3 l Erlenmeyer flasks containing 2.5 l medium were used and additional medium with 50 mM- NH_4Cl was pumped into the cultures using a calibrated peristaltic pump. The rate of medium addition was adjusted to compensate for ammonia uptake by the cells. Thus the ammonia content of the culture solution was maintained between 1.6 and 2.3 mM during the exponential growth phase.

^{15}N Ammonia incubation with and without inhibitors. The pretreated mycelia were resuspended in new culture medium (about 1 g fresh wt in 25 ml) containing 2 mM- NH_4Cl as sole nitrogen source. An equal volume of culture solution containing 2 mM- $^{15}\text{NH}_4\text{Cl}$ (99% enrichment: Amersham) was added to the resuspended mycelia. In the experiments with inhibitors, either AZS or PPT (sufficient to inhibit fungal growth) was first dissolved in the culture media with $^{15}\text{NH}_4\text{Cl}$ before being added to the resuspended mycelia, to give a final concentration of 2 or 5 mM, respectively. The culture media were aerated throughout the incubation period. The mycelia were harvested at intervals using a wide-mouth pipette and filtered through Miracloth. The filtrate was returned to the incubation flask in order to maintain a steady solute concentration in the incubation medium.

^{15}N analysis of nitrogen compounds. The nitrogenous compounds were extracted and separated as previously described (Kusnan *et al.*, 1987). The total nitrogen of mycelia was determined as ammonia following Kjeldahl digestion, micro-diffusion in Conway dishes and Nesslerization. ^{15}N enrichment was determined by emission spectroscopy as described previously (Berger & Fock, 1983). Appropriate corrections for isotope dilution arising from ammonia in the chemicals and gases used in the analytical procedure were made. Therefore, different concentrations of ^{15}N glutamate, ^{15}N glutamine and ^{15}N glycine (97, 96 and 95% enrichment, respectively; Amersham) were subjected to the same treatment as the amino acids of the sample for comparison. The percentage enrichment of ^{15}N amino acid standards varied with the amount of the amino acid analysed. The equation for the correction factor was established from these standard amino acids. The correction factor $y = 1/(0.000928x + 0.065)$ was then applied for each amino acid in the samples analysed, where x is the amount of amino acid analysed. To estimate ^{15}N enrichment of glutamine-amino and asparagine-amino, a portion of the basic/neutral amino acids was hydrolysed with 0.5 M-HCl at 100 °C for 1 h. Glutamate and aspartate formed from glutamine and asparagine were separated by ion-exchange chromatography as for the acidic amino acids. The labelling of the amido nitrogen of glutamine and asparagine was calculated algebraically from total glutamine and asparagine labelling and glutamine-amino and asparagine-amino, respectively.

Mathematical modelling. The mathematical modelling based on the equation derived by Rhodes *et al.* (1980) for 'steady-state' conditions was followed throughout.

$$dB^* = k_1QA^*dt - (k_1Q - afB)B^*dt$$

where ' Q ' is the quantity of cells which were growing exponentially with the specific growth rate ' a ', the compound ' B ' is derived from compound ' A ' with a transfer coefficient k_1 . If ' A ' is labelled, its fractional abundance being $A^* = A^*/A$, where A^* is the amount of labelled molecules in the total quantity ' A ', then ' B ' will be labelled to $B^* = B^*/B$. A 'steady-state' condition is considered as a special case where the correction factor $f = 1$.

Any model also includes an incremental increase in ' Q ' and ' B '

$$dQ = Qa \cdot dt$$

$$dB = Ba \cdot dt$$

The interaction time dt was set on 1 min intervals. The values of the model output were judged by a 'least-squares' fit.

RESULTS

^{15}N Ammonia assimilation under steady-state conditions

The mean of six replicate analyses of the total nitrogen content of mycelia during the incubation period was 345 $\mu\text{mol N}$ (100 mg dry wt tissue) $^{-1}$. The highest proportion of nitrogen was found in the ethanol-insoluble fraction, about 260 $\mu\text{mol N}$ (100 mg dry wt) $^{-1}$. The ethanol-

Fig. 1. ^{15}N uptake during $^{15}\text{NH}_4\text{Cl}$ feeding (43% enrichment). (a) ^{15}N enrichment of total nitrogen in *A. nidulans* and in various subfractions. The results are normalized to 100% $^{15}\text{NH}_4^+$. (b) ^{15}N content [$\mu\text{mol } ^{15}\text{N} (100 \text{ mg dry wt})^{-1}$] of the various fractions. $\circ$, Ethanol-soluble fraction; $\square$, ethanol-insoluble fraction; $\bullet$, total. The results are means of at least two replicate analyses.

soluble fraction contained only about $61 \mu\text{mol N} (100 \text{ mg dry wt})^{-1}$. The ^{15}N enrichment of the ethanol-soluble fraction increased rapidly to 19.7% within the first 30 min (Fig. 1a); then it was followed by a slower increment, which indicated that ammonia was assimilated into several different products in the ethanol-soluble fraction. The enrichment of the total and the ethanol-insoluble fractions increased steadily to 8.8 and 5.1%, respectively. Based on the mean total nitrogen and ^{15}N -enrichment of the total nitrogen about $218 \text{ nmol } [^{15}\text{N}]\text{ammonia} (100 \text{ mg dry wt})^{-1} \text{ min}^{-1}$ were assimilated (Fig. 1b).

The amounts of soluble nitrogen compounds in mycelia during incubation are shown in Fig. 2. An indication that a 'steady state' was achieved is given by the stability of the pools of individual amino acids. The small fluctuations observed are within the error of the analytical methods used. The pattern of ^{15}N labelling in the amino acids showed that glutamine-amide and glutamate were highly and almost equally labelled (Fig. 3a), while the labelling of alanine, glutamine-amino, aspartate and asparagine-amino (Fig. 3b), neutral and basic amino acids showed a lag phase (Fig. 3c, d). This may indicate that nitrogen enters the latter compounds only indirectly from intermediates that take some time to become fully labelled.

^{15}N assimilation in the presence of PPT or AZS

The effectiveness of the inhibitors used on ammonia assimilation in *A. nidulans* was indicated by the changes in the content of amino acids (Kusnan *et al.*, 1987) and the shift in the pattern of ^{15}N labelling in the major amino acids (Figs 4 and 5). In the presence of the GS inhibitor PPT, glutamate and alanine were the first highly labelled compounds while the labelling of glutamine and asparagine saturated at only 18.7% and 7.8% enrichment, respectively (Fig. 4a). Simultaneously, the level of [^{15}N]glutamate, the precursor of glutamine, and also that of [^{15}N]alanine greatly increased during the incubation period (Fig. 4b). This may indicate high flux rates of ^{15}N into glutamate and alanine during GS inhibition. The incorporation of ^{15}N at a low flux rate into glutamine (Fig. 4b) could be due to a residual GS activity before its complete inhibition by PPT. The effect of PPT on asparagine remains to be clarified, although it may be due to a decreased level of glutamine or inhibition of asparagine synthetase by PPT.

With AZS, the relative labelling of glutamate was very significant and higher than that of glutamine-amide (Fig. 5a). However, the absolute levels of [^{15}N]glutamate and [^{15}N]alanine remained low (Fig. 5b). The labelling of glutamine-amide was not saturated during 120 min of ^{15}N ammonia feeding (Fig. 5a). This might be explained by the large amount of unlabelled glutamine present as the consequence of the reduced utilization of glutamine via the inhibited GOGAT pathway. The labelling of glutamate is closely followed by that of alanine (see also Fig. 4a). The highly labelled glutamate can probably be used in the synthesis of alanine by the

Fig. 2. Amino acid levels in *A. nidulans* during $^{15}\text{NH}_4\text{Cl}$ feeding. The results of one experiment out of two replicates with similar trends are shown; the mean deviation from the values shown (of the means of replicate analyses) was about 10%. (a) ○, Glutamate; □, neutral amino acids; △, alanine; ■, glutamine; ●, neutral and basic fraction. (b) ○, Serine; □, aspartate; △, asparagine; ●, threonine. (c) ○, Valine; □, arginine; △, glycine; ●, lysine; ■, histidine.

Fig. 3. ^{15}N labelling of amino acids during $^{15}\text{NH}_4\text{Cl}$ feeding (43% enrichment). The data are normalized to 100% $^{15}\text{NH}_4^+$ in the medium. The experiment was repeated twice. Results of an individual experiment are shown. The trends of replicates were very similar, but absolute values were different. (a) ○, Glutamate; □, glutamine; ■, glutamine-amide; ●, glutamine-amino. (b) ○, Asparagine-amino; □, aspartate; △, alanine. (c) ○, Glycine; □, valine; ●, threonine; ■, serine. (d) ○, Histidine; □, lysine; ●, arginine; ■, neutral.

Fig. 4. Effect of 5 mM-PPT on (a) ^{15}N enrichment (%) of amino acids and (b) ^{15}N incorporated [μmol (100 mg dry wt) $^{-1}$] into amino acids during $^{15}\text{NH}_4\text{Cl}$ feeding (64% enrichment). The data are normalized to 100% $^{15}\text{NH}_4^+$ in the medium. The experiment was repeated twice. Results of an individual experiment are shown. The trends of replicates were very similar, but the absolute values were different. O, Glutamate; □, glutamine; △, alanine; ●, asparagine; ■, aspartate.

Fig. 5. Effect of 2 mM-AZS on (a) ^{15}N enrichment (%) of amino acids and (b) ^{15}N incorporated [μmol (100 mg dry wt) $^{-1}$] into amino acids during $^{15}\text{NH}_4\text{Cl}$ feeding (61% enrichment). The data are normalized to 100% $^{15}\text{NH}_4^+$ in the medium. The experiment was repeated twice. Results of an individual experiment are shown. The trends of replicates were very similar, but absolute values were different. O, Glutamate; □, glutamine-amide; △, alanine; ■, aspartate; ●, asparagine; ▲, glutamine-amino.

activity of aminotransferase. However, glutamine-amino, aspartate and asparagine were labelled at much slower rates.

DISCUSSION

^{15}N uptake

As judged from the amino acid pool sizes (Fig. 2) and the pattern of ^{15}N labelling (Fig. 3), glutamine-amide and glutamate seem to be the most important early products of ammonia assimilation in *A. nidulans* growing at low ammonia concentrations. However, the pattern of

^{15}N labelling in the major amino acids in this study differed from the pattern of ^{15}N labelling in major amino acids of ammonia assimilation by the GDH pathway in *Candida utilis* (Sims & Folkes, 1964), in which glutamate was initially more highly labelled than any other amino acid. Our results also differed from the pattern of labelling in the major amino acids of ammonia assimilation by the GS/GOGAT pathway in *Lemna minor* (Rhodes *et al.*, 1980) and barley roots (Fentem *et al.*, 1983), which showed that glutamine-amide was the first highly labelled compound.

The pattern of ^{15}N labelling in the presence of PPT is compatible with the operation of NADP-GDH in ammonia assimilation by *A. nidulans* (Fig. 4). NADP-GDH has been detected in this organism (Kusnan *et al.*, 1987; Pateman, 1969). It probably catalyses the direct formation of glutamate from ammonia and 2-oxoglutarate. Similar conclusions were made by Genetet *et al.* (1984) in experiments with *Cenococcum graniforme*, when this organism was incubated with [^{15}N]ammonia in the presence of MSO.

The notion that NADP-GDH may contribute to ammonia assimilation by *A. nidulans* is further supported by experiments with the GOGAT inhibitor AZS. The [^{15}N]glutamate synthesized in the absence (Fig. 3) and presence of AZS (Fig. 5) could be due to [^{15}N]ammonia assimilation via NADP-GDH, since the activity of NADP-GDH is not affected by AZS (Mifflin & Lea, 1975). The glutamate formed via the NADP-GDH pathway should be highly ^{15}N labelled, since its nitrogen is derived directly from [^{15}N]ammonia that has been fed.

The operation of the GS/GOGAT pathway in *A. nidulans* during growth at low ammonia concentration as suggested by Kusnan *et al.* (1987) was confirmed in this study. The glutamate presumably formed via GOGAT has a labelling comparable to the ^{15}N of amino acids which derive their nitrogen from an intermediate, i.e. glutamine. However, in the presence of AZS (an inhibitor of GOGAT; Fig. 5), the highly labelled glutamate formed via NADP-GDH was no longer diluted by lower labelled glutamate formed by GOGAT. In the presence of PPT the GS reaction was inhibited (Fig. 4a); consequently the glutamine pool declined (Fig. 4b) and glutamate synthesis via GOGAT was reduced. Because of changes in the content of amino acids and possibly keto acids (Kusnan *et al.*, 1987) glutamate formation via NADP-GDH may proceed, albeit at a lower rate.

Mathematical modelling of ^{15}N labelling

Estimates of the steady state fluxes of nitrogen through soluble amino acids in low-ammonia-grown *A. nidulans* were derived from the flux equation of Rhodes *et al.* (1980). Initially, the theoretical ^{15}N labelling curve for the amide group of glutamine was constructed and compared with the experimental data (Fig. 6a). The model is based on the assumptions (i) that no other route of glutamine formation exists except that through the amidation of glutamate (Pateman & Kinghorn, 1975), and (ii) that ammonia for amidation is solely derived from the internal ammonia pool which is the first to become labelled. The theoretical labelling curve for the free ammonia pool was constructed on the basis of a single ammonia pool of 1560 nmol (100 mg dry wt) $^{-1}$ the mean of six replicate analyses. The rate of ammonia uptake [517 nmol (100 mg dry wt) $^{-1}$ min $^{-1}$] was calculated from the rate of ^{15}N uptake (Fig. 1b). When an appropriate value for the transfer coefficient of ammonia into glutamine-amide [216 nmol (100 mg dry wt) $^{-1}$ min $^{-1}$] is chosen, the theoretical labelling curve closely fits the experimental data (Fig. 6a). The theoretical labelling curve for the ammonia pool is also shown in Fig. 6(a).

A theoretical curve was then constructed for ^{15}N in glutamate based on glutamate formation via the GDH pathway which derives its ammonia from the same pool as glutamine-amide (Fig. 6b). The theoretical curve fits the experimental data reasonably well, when the transfer coefficient is set at 208 nmol (100 mg dry wt) $^{-1}$ min $^{-1}$. However, this transfer coefficient for glutamate formation is too low to account for the observed rates of glutamine synthesis.

A model of glutamate formation based on the combined GDH and GOGAT pathways (Kusnan *et al.*, 1987) was constructed, assuming that, after subtraction of the GS contribution (above), the remainder of ammonia assimilation [301 nmol (100 mg dry wt) $^{-1}$ min $^{-1}$] occurs via the NADP-GDH pathway. In fitting the experimental data it was assumed that some glutamate

Fig. 6. Computer simulation of ammonia and glutamine (a) and glutamate (b) labelling. Lines are the results of computer simulation and points are the experimental data. (a) ----, Ammonia; $\square$, —, glutamine amide; $\circ$, —, total glutamine; $\blacksquare$, —, glutamine amino. (b) Glutamate formation directly via NADP-GDH with a transfer coefficient of 301 nmol (100 mg dry wt) $^{-1}$ min $^{-1}$ (----); glutamate formation (best fit) directly via NADP-GDH with a transfer coefficient of 208 nmol (100 mg dry wt) $^{-1}$ min $^{-1}$ (.....); glutamate formation via GOGAT with a transfer coefficient of 331 nmol (100 mg dry wt) $^{-1}$ min $^{-1}$ (.....); glutamate formation via the combined NADP-GDH and GOGAT pathways with transfer coefficients of 301 and 331 nmol (100 mg dry wt) $^{-1}$ min $^{-1}$, respectively (—).

originates from lower labelled glutamine via the GOGAT pathway. The theoretical labelling curves for glutamate, glutamine and glutamine-amino were then simultaneously constructed. The theoretical labelling curve based on the above assumption adequately fits the experimental data of glutamate (Fig. 6b), glutamine-amino and total glutamine labelling (Fig. 6a), when the transfer coefficient for glutamate synthesis via the GOGAT route was set at 331 nmol (100 mg dry wt) $^{-1}$ min $^{-1}$ which accounts for about 52% of the glutamate formed (Fig. 8).

In contrast, 10 to 20% of ammonia assimilation in *Candida utilis* was estimated to occur via pathways other than GDH and GS (Folkes & Sims, 1974). Thus in the model proposed, any reduction in the direct fixation of assimilated ammonia into glutamate via the GDH pathway will then be accompanied by an equivalent reduction in the contribution from the GOGAT pathway.

This, however, is only one possible model, in which the complicated processes of the living cells have been visualized as a single event. In several glutamine-requiring reactions, glutamine may act as the amido donor and glutamate is then released. However, its immediate fate is uncertain and this was not taken into consideration in the modelling. In the synthesis of glutamine in *C. utilis* part of the glutamate consumed was postulated to be derived from the breakdown of glutamine itself (Folkes & Sims, 1974). Thus the synthesis of amino acids which utilize glutamate formed from glutamine will be underestimated, since only highly labelled glutamate was used in the simulation.

We also examined the possibility of a separate glutamine pool by testing the [^{15}N]glutamine-amide data by the model. The theoretical labelling curve for glutamine-amide could also closely fit the experimental data, when 4 to 7% of glutamine was present in a storage pool. In the presence of PPT the pool of glutamine was rapidly depleted (Fig. 4b), suggesting that glutamine was mainly in the actively metabolized pool (Kusnan *et al.*, 1987). However, the scatter of the ^{15}N data was too high to allow a more detailed analysis and construction of better-fitted curves for glutamine and glutamate.

The theoretical labelling curve was then constructed for other amino acids on the basis of the established routes of amino acid biosynthesis (Pateman & Kinghorn, 1975; Umbarger, 1978); the results are shown in Fig. 7. Except for arginine, the theoretical labelling curves satisfactorily fit the experimental labelling data based on single pool kinetics.

In addition to glutamate, alanine could also play an important role as an amino donor, because of its large pool size and high rates of labelling and utilization (Figs 3, 4 and 5; Kusnan *et al.* 1987); Fig. 8 probably underestimates its importance. However, since the mechanism of

Fig. 7. Computer simulation of labelling of amino acids. Lines represent the computer simulation labelling and points are the experimental data. (a) ●, —, Aspartate; □, ----, asparagine-amino; (b) ●, —, alanine; □, ----, glycine; (c) histidine; (d) threonine; (e) valine; (f) lysine; (g) serine; (h) neutral; (i) arginine (middle line resultant of mean, upper line of pool I and lower line of pool II).

transamination is very flexible and almost any amino acid can act as amino donor (Fincham & Boulter, 1956), the role of alanine as amino donor could not be assessed in this study.

In the synthesis of glycine, the alanine:glyoxylate aminotransferase pathway was followed. Thus the amino group of glycine was derived from alanine (Galbraith & Smith, 1969), rather than by conversion of serine by the serine hydroxymethyl transferase pathway (for review see Pateman & Kinghorn, 1975). This was deduced from the fact that the transfer coefficient of serine was too small to account for the formation of glycine.

The transfer coefficient of aspartate is much lower than the combined transfer coefficients for the formation of amino acids which require aspartate, including arginine (Fig. 8). The aspartate that was analysed in the acid hydrolysate of the ethanol-insoluble fraction could also be the product of acid hydrolysis of asparagine. Furthermore, another route for asparagine formation has been shown to occur in *Neurospora crassa* in which α -ketosuccinamate is aminated with glutamine by asparagine aminotransferase to yield asparagine (Monder & Meister, 1958).

The theoretical labelling curve of arginine fits the experimental labelling data best, when it is assumed that 55% of the total pool was not actively turning over. This estimate is much lower than the estimated storage pool in *N. crassa*, in which 94% of the arginine was located in the vesicles (Weiss, 1973). Our estimate of the active arginine pool is higher; this may be due to partial hydrolysis of nitrogen groups other than α -amino.

According to this simple model, about 42% of the newly assimilated ammonia was fixed into the amide group of glutamine. Of the total glutamate 52% was formed via the GOGAT pathway from recently synthesized glutamine and mixed with glutamate formed via the NADP-GDH pathway: thus kinetically only a single glutamate pool exists. The net formation of glutamate via the NADP-GDH (48%) and GOGAT (52%) pathways in the model is higher than the estimated glutamate requirement (Fig. 8). However, due to lack of labelling data of several other amino acids including proline and γ -ABA, the transfer coefficients of these amino acids could not be determined.

The results of this study clearly indicate that the concurrent operation of the NADP-GDH and GS/GOGAT pathways in ammonia assimilation by *A. nidulans* differs from the previous observations on *Lemna minor* and barley roots (Fentem *et al.*, 1983; Lewis *et al.*, 1983; Rhodes *et al.*, 1980). Although substantial activity of NADP-GDH was detected in these plants, ammonia was assimilated solely by the GS/GOGAT pathway.

Fig. 8. Summary of the transfer coefficients of ammonia into amino acids [—; $\text{nmol (100 mg dry wt)}^{-1} \text{ min}^{-1}$] in the labelling experiment with $[^{15}\text{N}]$ ammonia. Numbers in boxes are the pool sizes [$\mu\text{mol (100 mg dry wt)}^{-1}$]. ---, Rate of amino acid formation in the ethanol-insoluble fraction after hydrolysis with 6 M-HCl (Perham, 1978) and calculated to maintain exponential-phase growth [$\text{nmol (100 mg dry wt)}^{-1} \text{ min}^{-1}$]. Numbers in circles are the calculated rates to maintain pool sizes during exponential-phase growth [$\text{nmol (100 mg dry wt)}^{-1} \text{ min}^{-1}$]. * Associated with γ -ABA peak; ? glutamine and asparagine were determined as glutamate and aspartate; ND, not determined.

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