Effect of Methionine Sulfoximine on Asparaginase Activity and Ammonium Levels in Pea Leaves

Konrad A. Sieciechowicz, Kenneth W. Joy*, and Robert J. Ireland

Department of Biology, Carleton University, Ottawa, Ontario K1S 5B6, Canada (K.A.S., K.W.J.); and Department of Biology, Mount Allison University, Sackville, New Brunswick E0A 3C0, Canada (R.J.I.)

ABSTRACT

In developing leaves of Pisum sativum the levels of ammonium did not change during the light-dark photoperiod even though asparaginase (EC 3.5.1.1) did; asparaginase activity in detached leaves doubled during the first 2.5 hours in the light. When these leaves were supplied with 1 millimolar methionine sulfoximine (MSX, an inhibitor of glutamine synthetase, GS, activity) at the beginning of the photoperiod, levels of ammonium increased 8- to 10-fold, GS activity was inhibited 95%, and the light-stimulated increase in asparaginase activity was completely prevented, and declined to less than initial levels. When high concentrations of ammonium were supplied to leaves, the light-stimulated increase of asparaginase was partially prevented. However, it was also possible to prevent asparaginase increase, in the absence of ammonium accumulation, by the addition of MSX together with aminoxyacetate (AOA, which inhibits transamination and some other reactions of photorespiratory nitrogen cycling). AOA alone did not prevent light-stimulated asparaginase increase; neither MSX, AOA, or elevated ammonium levels inhibited the activity of asparaginase in vitro. These results suggest that the effect of MSX on asparaginase increase is not due solely to interference with photorespiratory cycling (since AOA also prevents cycling, but has no effect alone), nor to the production of high ammonium concentration or its subsequent effect on photosynthetic mechanism. MSX must have further inhibitory effects on metabolism. It is concluded that accumulation of ammonium in the presence of MSX may underestimate rates of ammonium turnover, since liberation of ammonium from systems such as asparaginase is reduced by the effects of MSX.

The degradation of asparagine is important in supplying nitrogen for amino acid and protein synthesis in developing leaves, and N-labeling studies have indicated that up to 75% of the nitrogen required for growth in half-expanded pea leaves was derived from the amide-nitrogen of asparagine (25). The amide-nitrogen of asparagine can be liberated directly by asparaginase (EC 3.5.1.1.) (8), producing aspartate and ammonium, or by deamination of products (2-oxosuccinamyl acid [14] or hydroxysuccinimide [25, 26]) resulting from asparaginase transamination (asparagine: pyruvate transaminase, EC 2.6.1.14). In developing pea leaves, asparaginase activity undergoes diurnal variation, increasing in the light (20), a process dependent upon photosynthetic electron tran

1 Supported by grants to K. W. J. and R. J. I. from Natural Sciences and Engineering Research Council of Canada.
2 Current address: Department of Biological Sciences, Simon Fraser University, Burnaby, B. C. Canada, V6A 1S6.
3 Abbreviations: AOA, aminoxyacetate; GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; MSX, methionine sulfoximine.
METHIONINE SULFOXIMINE AND ASPARAGINASE ACTIVITY

MATERIALS AND METHODS

Nonnodulated Pisum sativum L. (cv Little Marvel No. 1; McKenzie Steele Briggs, Brandon, Manitoba, Canada) plants were grown with 16 h of light and 8 h of dark (18°C), as reported earlier (21). Half-expanded fifth leaves were used for all enzyme extractions and feeding studies (18–20 d after seed imbibition).

All biochemicals were obtained from Sigma Chemical Co., (St. Louis, MO.).

Enzyme Assays

Asparaginase and glutamine synthetase (biosynthetic assay) activities were assayed as reported earlier (20), except that 1 mM CaCl₂ and 10% glycerol were added to the asparaginase extraction and assay buffers.

The assay for NADH glutamate dehydrogenase was adapted from Pahlich and Joy (16). Leaf tissue was extracted and desalted (Sephadex G 25, equilibrated in the same buffer) in 50 mM Tris HCl (pH 7.8) containing 10 mM MgSO₄, 1 mM EDTA, and 1 mM DTT. For assaying reductive amination of oxoglutarate, 0.1 mL aliquots of the extract were added to mixtures to give final concentrations of 50 mM Tris HCl, 1 mM CaCl₂, 13 mM oxoglutarate, and 60 mM (NH₄)₂SO₄. Reactions were started by the addition of 0.3 mM NADH (total assay volume 1.2 mL), and the decreases in A₅₄₀ were followed over a 5 min period at 25°C. Controls, without enzyme or substrates, were also run with both enzyme assays.

Analysis of Ammonium

The levels of ammonium in leaf extracts were analyzed by either microdiffusion followed with nesslerization (19), or with a Beckman 119BL amino acid analyzer (physiological mode).

Feeding Experiments

Just before the end of the dark period, or after 14 h of the light period, whole shoots were excised above the cotyledon and quickly transferred to distilled water. Petioles of half-expanded leaves were then cut under water just above the stipules and placed individually in small tubes containing 0.3 mL of distilled water or 8.0 mM asparagine, with 1 mM MSX and/or 4 mM AOA, or 10 to 100 mM NH₄Cl (all at pH 6.3).

Ten replicates were used per treatment. Control samples were examined for ammonium levels, and/or asparaginase and glutamine synthetase activities at the start of the feeding experiment, and the remaining leaves were placed in growth cabinets and illuminated with a combination of incandescent and fluorescent sources (285 μmol m⁻² sec⁻¹ PAR, see above) for 2.5 h.

RESULTS AND DISCUSSION

The deamidation of asparagine in developing pea leaves is catalyzed by asparaginase, in a reaction producing aspartate and ammonium. Other processes are also known to liberate ammonium in leaf tissues: the oxidation of glycine results in photorespiratory ammonium release; nitrate reductase and NiR activities reduce inorganic nitrogen to the level of am- monium; phenylalanine and tyrosine ammonia lyases, threonine dehydratase, other deamidases (e.g. glutaminase, and ω-amidase), and urease produce ammonium; ammonium is also liberated during protein turnover.

Several of the above processes produce more ammonium during the light (e.g. asparaginase, photorespiration, phenylalanine ammonia lyase, nitrate reductase, nitrite reductase) than in the dark, yet when the levels of ammonium in half-expanded pea leaves were monitored over a 24 h photoperiod, they remained low (0.8–1.5 μmol NH₃ g fresh weight⁻¹), with only a slight increase in the light (Fig. 1). The importance of GS-GOGAT in the assimilation for ammonium into organic form in plant tissues has received much support over the past few years, and it has been suggested that preference of this pathway over the assimilation of ammonium by GDH depends on the relative affinities of GS and GDH for ammonium (24). The levels of GS and GDH were followed throughout the photoperiod. The activity of GS remained high, although it displayed an ultradian rhythm, similar to that found in sunflower roots (11), while GDH activity decreased to quite low levels in the light (Fig. 1) as found in some other tissues (23). These results support the role of GS as the primary scavenger of cellular ammonium, and suggest that GS has excess capacity for the maintenance of low ammonium levels within the cell.

Concentrations of MSX from 1 to 10 mM have been used to demonstrate the involvement of GS in the assimilation of ammonium derived from photorespiration in C₃ and C₄ leaf tissues (1, 10, 13), and 1 to 4 mM AOA (in the presence of MSX) to indicate that transamination is important in introducing nitrogen into the photorespiratory pathway (13, 29). Similar concentrations of MSX and AOA have been used to
indicate that transamination precedes deamidation in the liberation of ammonium from asparagine in pea leaves (8, 25).

Since asparaginase activity increased substantially in extracts of light-harvested leaves (20, 21), we wished to determine whether this resulted in a more rapid rate of deamidation, in vivo, of asparagine. Half-expanded leaves were fed with water or asparagine solutions containing MSX to inhibit GS activity and thus prevent the assimilation of asparaginase-derived ammonium. Other treatments included feeding AOA (with or without MSX) to reduce the production of photorespiratory ammonium.

Patterns of ammonium accumulation observed in MSX, AOA, or MSX+AOA feeding treatments were similar for leaves treated with or without addition of asparagine, although levels were higher in the presence of asparagine (Table I). Half-expanded leaves obtained at the beginning or end of the light period displayed increased ammonium levels when supplied with MSX and this result is consistent with the role of glutamine synthetase in the assimilation of ammonium in leaf tissues. The addition of AOA alone to feeding solutions did not greatly alter the levels of ammonium within leaves, yet when AOA was added together with MSX, the high values of ammonium observed in the presence of MSX were reduced and were only slightly higher than those of the control treatments (Table I). These results suggest that AOA-sensitive reactions (presumably transamination, involved in photorespiration) are responsible for production of most, if not all, of the ammonium which accumulates in the leaf when GS is inhibited. Yamaya and Matsumoto (29) and Martin et al. (13) have also observed similar trends in the levels of ammonium in the presence of MSX and AOA.

Since asparaginase levels are three- to fivefold higher after 14 h of light (21), much higher ammonium levels were expected in the leaves fed MSX or MSX+AOA at the end of the light period compared with those fed at the end of the dark. This was not observed (Table I) and suggested that either the production of ammonium by asparaginase is insignificant in comparison with the release from photorespiration, or that the inhibitor used interfered with asparaginase activity. Since increased asparaginase activity in the light requires photosynthetic electron transport (21), and MSX has been reported to inhibit photosynthesis (see below), the effect of MSX on asparaginase activity was investigated.

Neither MSX nor AOA had any direct inhibitory effect on asparaginase activity in vitro (Table II). The in vivo effect was investigated by supplying asparaginase together with a range of MSX concentrations to detached half-expanded leaves for 2.5 h in light followed by the assay of GS and asparaginase activities, and ammonium levels. As expected, GS activity was reduced by over 90% and an eight- to ninefold increase in the levels of ammonium was also observed (Fig. 2a), confirming that GS was inhibited in the presence of MSX. Over the 2.5 h feeding period in the light, increased asparaginase activity (twofold) was observed in the leaves supplied with asparagine, but asparaginase activity in extracts obtained from the MSX treated leaves decreased below that of the 0 h control (Fig. 2a). The loss of asparaginase activity in detached leaves supplied with MSX was similar to the decrease in activity noted when detached leaves were fed asparagine alone in the dark for 2.5 h (21). Since MSX had no effect on asparaginase in vitro, and asparaginase is affected by photo- synthetic electron transport (21, 22), decreased asparaginase levels in the light during MSX treatment may be a result of secondary effects of MSX on photosynthetic activity.

Increased ammonium levels caused by the presence of MSX could account for prevention of the increase in asparaginase activity, although asparaginase activity in vitro was not inhibited by the addition of high (up to 0.1 M) concentrations of NH₄Cl (Table II). When detached, half-expanded leaves were supplied with ammonium the increase in asparaginase was unaffected at low ammonium concentrations, with some progressive effect noted at higher concentrations (Fig. 2b). However, the increase in asparaginase was completely abolished only with the supply of 50 mM ammonium, producing internal concentrations much higher than that caused by MSX.

### Table I. Effects of MSX and AOA on Ammonium Accumulation in Half-Expanded Pea Leaves

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 h ± sD</th>
<th>14 h ± sD</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>1.61 ± 0.2</td>
<td>1.29 ± 0.3</td>
</tr>
<tr>
<td>+ MSX</td>
<td>8.44 ± 0.8</td>
<td>8.60 ± 1.0</td>
</tr>
<tr>
<td>+ AOA</td>
<td>1.04 ± 0.2</td>
<td>0.91 ± 0.1</td>
</tr>
<tr>
<td>+ MSX + AOA</td>
<td>1.78 ± 0.4</td>
<td>1.98 ± 0.2</td>
</tr>
<tr>
<td>Asn</td>
<td>0.97 ± 0.3</td>
<td>0.83 ± 0.2</td>
</tr>
<tr>
<td>+ MSX</td>
<td>14.09 ± 0.7</td>
<td>10.70 ± 0.3</td>
</tr>
<tr>
<td>+ AOA</td>
<td>1.33 ± 0.3</td>
<td>0.75 ± 0.5</td>
</tr>
<tr>
<td>+ MSX + AOA</td>
<td>1.76 ± 0.3</td>
<td>1.60 ± 0.2</td>
</tr>
</tbody>
</table>

### Table II. Effects of MSX, AOA, and Ammonium on in Vitro and in Vivo Asparaginase Activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>In Vitro Activity</th>
<th>In Vivo Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% control activity</td>
<td>% 2.5 h activity ± sD</td>
</tr>
<tr>
<td>Control (no additions)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+ MSX 0.5 mM</td>
<td>102</td>
<td>26</td>
</tr>
<tr>
<td>+ MSX 1.0 mM</td>
<td>96</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>+ AOA 1.0 mM</td>
<td>5.0</td>
<td>98</td>
</tr>
<tr>
<td>+ MSX + AOA 2.5 h in the light (in vivo activity)</td>
<td>101</td>
<td>98</td>
</tr>
<tr>
<td>+ NH₄ 0.01 M</td>
<td>4.0</td>
<td>99</td>
</tr>
<tr>
<td>+ NH₄ 0.1 M</td>
<td>100</td>
<td>30 ± 7</td>
</tr>
</tbody>
</table>

* MSX and AOA concentrations were 1.0 and 4.0 mM, respectively."
AOA (9), supporting photorespiration are ammonium port. It might be due to the reduction of MSX, inase might be the decrease to A OA (17, 27), nor does MSX inhibit photosynthesis in isolated chloroplasts (15). However, other mechanisms for the inhibition of photosynthesis by MSX may exist.

AOA reduces the flux of photorespiratory intermediates (9), yet in the presence of AOA the increase of asparaginase was only slightly less than in the control (Table II). Furthermore, in the presence of AOA and MSX together, ammonium levels were not elevated (Table I), yet the light-stimulated increase of asparaginase was prevented (Table II). This suggests that neither inhibition of carbon recycling, nor the increased ammonium concentration are the principle cause of the MSX effect on asparaginase increase, and thus MSX must be suspected of an additional, unidentified inhibitory effect on metabolism.

Results obtained by using MSX + AOA (13, 29), MSX + isonicotinyl hydrazide (12, 13, 27), or MSX + amino-acetonitrile (1) have been presented as support for the idea that the major source of ammonium in leaves (an order of magnitude higher than primary assimilation [10]) is from photorespiration. Similar conclusions have also been reported from studies using only MSX (2, 7, 10, 17). However, decrease of asparaginase activity in vivo by MSX suggests that estimates of ammonium levels in leaves fed with MSX are not representative of ammonium production from all sources, although photorespiratory ammonium production appears to be unaffected by MSX (10; however, see Ref. 27). If there is a similar effect on other light-requiring ammonia-liberating enzymes, the discrepancy could even be greater.

The addition of MSX and AOA have also been used to demonstrate that flow of nitrogen from asparagine in half-expanded pea leaves is through the deamidation of 2-oxosuccinamic acid, the transamination product of asparagine, or its subsequent reduction product, hydroxysuccinamic acid, rather than through the deamination of asparagine (8, 25, 26). In view of the decrease in asparaginase activity during MSX feeding experiments, these results may underestimate the contribution of asparaginase in vivo.

The results lend further support to the observations of others on the secondary effects of MSX on cellular metabolism (1, 7, 9, 17, 27), and indicate that MSX is not a reliable inhibitor for the estimation of ammonium production in vivo as had been widely assumed.

LITERATURE CITED


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