Adenylate Gradients and Ar:O₂ Effects on Legume Nodules. II. Changes in the Subcellular Adenylate Pools

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Central infected zone tissue of soybean (Glycine max L. Merr.) nodules was fractionated into separate subcellular compartments using density gradient centrifugation in nonaqueous solvents to better understand how exposure to Ar:O₂ (80:20%, v/v) atmosphere affects C and N metabolism, and to explore a potential role for adenylates in regulating O₂ diffusion. When nodules were switched from air to Ar:O₂, adenylate energy charge (AEC) in the plant cytosol rose from 0.63 ± 0.02 to 0.73 ± 0.02 within 7 min and to 0.80 ± 0.01 by 60 min. In contrast, AEC of the mitochondrial compartment of this central zone tissue remained high (0.80 ± 0.02 to 0.81 ± 0.02) following Ar treatment while that of the bacteroid compartment was unchanged, at 0.73 ± 0.02, after 7 min, but declined to 0.57 ± 0.03 after 60 min. These results were consistent with a simulation model that predicted Ar:O₂ exposure would first reduce ATP demand for ammonia assimilation and rapidly increase cytosolic AEC, before the Ar:O₂-induced decline mediated by a decrease in nodule O₂ permeability reduces bacteroid AEC. The possibility that adenylates play a key, integrating role in regulating nodule permeability to oxygen diffusion is discussed.

In active, N₂ fixing nodules, respiration and N₂ase activity are O₂ limited and can be increased slightly, but significantly (2%–20%), by gradual increases in the external partial pressure of O₂ (pO₂; Hunt et al., 1989). However, following treatments such as defoliation, detopping, stem girdling, nitrate fertilization, or exposure to C₂H₂ or Ar:O₂ that are known to inhibit nitrogenase activity and nodule respiration, nodules respond to increases in pO₂ with much larger enhancements of nitrogenase activity (Hartwig et al., 1987; Vessey et al., 1988; Denison et al., 1992; de Lima et al., 1994). These studies, together with measurements of infected cell O₂ concentration and nodule permeability (Denison and Layzell, 1991; King and Layzell, 1991), showed that the treatments apparently down-regulate metabolism by reducing the supply of O₂ through a decrease in the gaseous permeability of nodules.

The mechanism that nodules use to regulate the diffusion barrier is unknown, but is correlated with the adenylate ratios (ATP-ADP; adenylate energy charge, \( AEC = ([ATP] + 0.5[ADP]) / ([ATP] + [ADP] + [AMP]) \)) in most, but not all treatments known to induce severe O₂ limitation of nodule metabolism (de Lima et al., 1994). For example, exposure of nodules to 10% O₂ (2–3 min), stem girdling (3 h), or nitrate fertilization (48 h) decreased nodule AEC values by approximately 0.12 units (de Lima et al., 1994). However, 60 min exposure of soybean (Glycine max) nodules to Ar:O₂ did not change the nodule AEC values (de Lima et al., 1994), even though the treatment dramatically lowered infected cell [O₂] (King and Layzell, 1991), and N₂ase activity could be recovered at elevated pO₂. Taken at face value, this result does not support the idea that adenylate pools or adenylate ratios in the infected cells are involved in the mechanism of O₂ regulation and diffusion barrier control in legume nodules.

Nevertheless, adenylates do seem to play a key role in the regulation of nodule metabolism. Kuzma et al. (1999) used a nonaqueous fractionation technique to show that bacteroids were the primary site of O₂ limitation in legume nodules, despite the fact that the AEC of the plant cytosol was much lower (AEC = 0.65 ± 0.04) than that expected for fully aerobic tissues (AEC > 0.80). To explain why the cytosolic AEC was so low, but unaffected by treatments that impose an O₂ limitation on nodules, Wei et al. (2004) generated a model of O₂ and adenylate gradients across the bacteria-infected cell. The model took account of the fact that mitochondria (site of ATP synthesis) in the infected cells were clustered near the intercellular spaces, whereas the sites of ATP use in the cytosol were predominantly within the bacteroid zone of the infected cells. In this simulation ATP utilization was associated with the activities of glutamine synthetase (GS) and asparagine synthetase in assimilating fixed N, and with the cost of transporting C₄ acids across the symbiosome membrane. The large distances between

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sites of ATP production and use were predicted to generate steep gradients in ATP, ADP, and AMP in the cytosol of the infected cell (Wei et al., 2004).

The model also predicted that short-term exposure of nodules to an Ar:O2 atmosphere—a treatment that stops ATP demand for N assimilation by GS and asparagine synthetase activities, but not nitrogenase activity—would increase the infected cell’s average cytosolic AEC from 0.70 to 0.77. If this prediction was correct, then the work of de Lima et al. (1994) showing that Ar:O2 does not affect whole-nodule AEC could be explained by an increase in cytosolic AEC that obscures an O2-limited decrease in bacteroid AEC that should occur with longer term exposure to Ar:O2. Such a finding would be consistent with the possibility that infected cell adenylate pools may, in fact, be involved in the regulation of the oxygen diffusion barrier.

This study uses nonaqueous centrifugal density gradient fractionation of the central infected zone tissue of soybean nodules to permit reliable recovery of adenylate pools from subcellular compartments. The nonaqueous technique is particularly useful for legume nodules since their metabolism is highly compartmentalized, not only within cells, but also between cell and tissue types (Smith and Atkins, 2002). This technique allowed a test of the hypothesis that Ar:O2 exposure would increase the cytosolic AEC in the central infected zone while decreasing the AEC in bacteroids.

RESULTS

Ar:O2 Treatment and Nitrogenase Activities

Out of three uniform populations of soybean plants, 18 were harvested after the N2:O2 (control) treatment, 18 after the 7-min Ar:O2 treatment, and 17 after the 60-min Ar:O2 treatment. Since no significant variations were observed in the measured apparent (ANA) and total (TNA) nitrogenase activities among plants destined for different treatments, the data were pooled for presentation in Figure 1A. The average ANA of nodules of plants selected for nonaqueous fractionation was 87 ± 9 μmol H2 g−1 DW(nod) h−1 (n = 18 + 18 + 17 = 53; Fig. 1A). After 7 min Ar:O2 exposure, H2 production increased to 240 ± 10 μmol H2 g−1 DW(nod) h−1 (TNA), resulting in an electron allocation coefficient (EAC = 1 − ANA/TNA) of 0.64 ± 0.03 (n = 18 + 17 = 35).

TNA declined sharply after 7 min in Ar:O2 and then recovered slightly and declined more gradually to 123 ± 8 μmol H2 g−1 DW(nod) h−1 (n = 17) after 60 min (Fig. 1A). This value was 51 ± 2% (n = 17) of the TNA value measured after 7 min Ar:O2 exposure.

Ar:O2 Treatment and Nodule Adenylate Pools

To permit direct comparison of adenylate concentrations between different nodule tissues, all the measured values were expressed in μmol g−1 DW(nod) (Fig. 1, B–D). Consistent with an earlier study (Oresnik and Layzell, 1994), the majority (94%) of adenylates in the whole nodule were in the central zone (CZ), whereas only 6% were in the cortex.

Ar:O2 exposure had no significant effect on total adenylate contents in either whole frozen nodules or dissected nodule CZ and cortex tissues (Fig. 1, B–D). The measured total adenylate pool in the frozen nodule averaged 1.8 ± 0.1 μmol adenylate g−1 DW(nod) (Fig. 1B).

The AEC in frozen nodule tissues increased from 0.73 to 0.76 within 7 min Ar:O2 exposure but then declined to 0.72 after 60 min Ar:O2 exposure (Fig. 1B). The same trend was found for AEC of the CZ tissue under Ar:O2 treatment (Fig. 1C). In contrast, AEC in the cortex was 0.83 to 0.84 and was unchanged during Ar:O2 exposure (Fig. 1D).
To assess adenylate losses following lyophilization and subsequent dissection, recovery was calculated as the sum of total adenylate in CZ and cortex (μmol g⁻¹ DW(nod), Fig. 1, C–D), divided by the total adenylate in frozen whole nodules (μmol g⁻¹ DW(nod), Fig. 1B). Recovery of total adenylate was 85 ± 3%, 84 ± 2%, and 82 ± 2% (n = 3 in each case) for tissue samples from the N₂O₂, 7-min Ar:O₂, and 60-min Ar:O₂ treatments, respectively.

The protein content of frozen nodule tissues was 127 ± 6 mg g⁻¹ DW(nod) (n = 9), whereas values for CZ and cortex tissues were 111 ± 5 and 11 ± 1 mg g⁻¹ DW(nod) (n = 9), respectively. Therefore, recovery of protein following lyophilization and subsequent dissection was 96%.

Distribution Patterns of Marker Enzyme Activities and Adenylates Following Nonaqueous Fractionation of Sonicated Nodule Tissues

There were no significant differences in the proportional distribution of protein or marker enzyme activities in the nonaqueous gradient fractions collected following centrifugation of sonicated tissues from nodules exposed to N₂O₂, or after 7 or 60 min Ar:O₂. Therefore, the data obtained from nine nonaqueous treatments (3 treatments × 3 replicates per treatment) were pooled to calculate the proportional distribution in each of the fractions (Fig. 2). Hydroxypyruvate dehydrogenase (HBD), the marker enzyme for bacteroids, was mainly localized in the top five fractions of the density gradient, whereas glutamate dehydrogenase (GDH; mitochondrial marker) and phosphoenolpyruvate carboxylase (PEPC; plant cytosolic marker) were more widely distributed and showed additional peaks of activity in gradient fractions 7 to 9 (Fig. 2A). This pattern of fractionation was broadly similar to that in an earlier study (Kuzma et al., 1999).

Adenylates in each of the 10 fractions were also measured, and the results for the three treatments are shown in Figure 3. Only small differences in the relative ATP and ADP distributions were observed between the treatments, whereas the differences were more pronounced in the distribution of AMP across gradient fractions (Fig. 3).

To assess the effect of sonication on the recovery of soluble protein, total adenylate (ATP + ADP + AMP), and activities of marker enzymes, the sonicated homogenate before fractionation was assayed, and the measured values are expressed as a percentage of the amount in the lyophilized CZ tissue (Table I). Recovery of soluble protein, total adenylate, and activities of PEPC and GDH after sonication were close to 100%; however, HBD activity recovered after sonication was 149 ± 6% of that in the lyophilized CZ tissue (Table I), consistent with the observation of Kuzma et al. (1999), who noted that sonication in nonaqueous solvents released more HBD activity than extraction in aqueous buffer. In addition, to assess the overall effect of sonication and the subsequent nonaqueous fractionation on the recovery of metabolites and activities of marker enzymes, the total of all values in the 10 fractions are expressed as a percentage of those in the lyophilized CZ tissue, and the results are presented in Table I.

Estimation of Subcellular Adenylate Pools under N₂O₂ and Ar:O₂ Treatments

Combining the results for adenylate assays (Fig. 3) with the marker enzyme distribution for each fraction (Fig. 2) and using the Bestfit program described by Riens et al. (1991), values were derived for the pool sizes of ATP, ADP, and AMP in the bacteroids, plant cytosol, and mitochondria (Fig. 4). These calculations were based on the assumption that the adenylates in the infected cells of soybean nodules were localized in one of the three compartments and that the distribution of marker enzymes reflected the proportional distribution of the contents of these compartments.

In bacteroids, the ATP concentrations remained unchanged within 7 min Ar:O₂ exposure, but declined to 78% of the initial level after 60 min in Ar:O₂ (Fig. 4A). While the ADP levels in bacteroids were not significantly changed during Ar:O₂ exposure, the AMP levels increased sharply by nearly 6-fold between 7 and 60 min Ar:O₂ exposure (Fig. 4A).

In plant cytosol, the ATP concentrations increased to 123% of the initial level following 7 min Ar:O₂ exposure, and to 131% of the initial level after 60 min in Ar:O₂, whereas the ADP and AMP levels declined gradually (Fig. 4B).
In the mitochondrial compartment, there were no significant changes in the levels of ATP, ADP, or AMP after 7 and 60 min Ar:O₂ exposure (Fig. 4C).

The total adenylate pools in bacteroids, plant cytosol, and mitochondria were 6.2 ± 0.4, 6.4 ± 0.4, and 1.4 ± 0.2 nmol mg⁻¹ CZ protein, respectively. They accounted for 45 ± 2%, 45 ± 2%, and 10 ± 2%, respectively, of total adenylate in the infected cells. The summed percentage of adenylate pools in the plant compartment (plant cytosol + mitochondria) was 54 ± 2%, similar to that reported in an earlier study (Kuzma et al., 1999).

Effect of Ar:O₂ on Adenylate Ratios in Subcellular Compartments of Nodules

The results of Figure 4 were used to determine the effect of Ar:O₂ exposure on AEC and ATP-ADP ratio in the bacteroids, cytosol, and mitochondria of the CZ tissue (Fig. 5). Within 7 min Ar:O₂ exposure, cytosolic AEC increased significantly from 0.63 ± 0.02 in N₂:O₂ to 0.73 ± 0.02 (0.05 > P > 0.02), and increased further to 0.80 ± 0.01 after 60 min Ar:O₂ exposure (0.002 > P > 0.001; Fig. 5A). In contrast, AEC in the bacteroid compartment was unchanged (0.73 ± 0.02 in N₂:O₂) after 7 min Ar:O₂ exposure, but then declined to 0.57 ± 0.03 after 60 min (0.01 > P > 0.002; Fig. 5A). In mitochondria, AEC was high under N₂:O₂ treatment (0.80 ± 0.02) and remained high following exposure to 7 and 60 min Ar:O₂ (0.81 ± 0.02, 0.81 ± 0.02; Fig. 5A).

The AEC of the whole CZ tissue calculated as the sum of the AEC values for all three compartments was 0.70 ± 0.02, 0.74 ± 0.02, and 0.70 ± 0.02 for the N₂:O₂, 7-min, and 60-min Ar:O₂ treatments, respectively (Fig. 5A). These values were similar to those measured in the lyophilized CZ tissue before fractionation (Fig. 1C).

The calculated subcellular ATP-ADP ratios for the three compartments showed similar trends to those observed for AEC (Fig. 5B).

DISCUSSION

This study is, to our knowledge, only the second report (after Kuzma et al., 1999) that describes the use of a nonaqueous fractionation technique to study changes in metabolite pools in subcellular compartments of legume nodules.

Unlike the previous report with soybean nodules (Kuzma et al., 1999), this study achieved a separation of the main peak of mitochondria (represented by the marker enzyme GDH; Fraction 8 in Fig. 2A) from that of plant cytosol (represented by the marker enzyme PEPC; fraction 8 in Fig. 2A). Similar separations have been obtained in spinach (*Spinacia oleracea*) leaf tissues (Gerhardt and Heldt, 1984; Weiner et al., 1987). This subcellular fractionation was an improvement over

Table I. Recovery of soluble protein, total adenylates, and marker enzyme activities during sonication and nonaqueous fractionation of lyophilized CZ tissue

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total Adenylate</th>
<th>HBD</th>
<th>PEPC</th>
<th>GDH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg g⁻¹ DW tissue</td>
<td>nmol mg⁻¹ Tissue prot</td>
<td>nmol min⁻¹ mg⁻¹ Tissue prot</td>
<td>n mol min⁻¹ mg⁻¹ Tissue prot</td>
</tr>
<tr>
<td>Lyophilized CZ</td>
<td>160 ± 7</td>
<td>12.7 ± 0.9</td>
<td>43 ± 2</td>
<td>310 ± 20</td>
</tr>
<tr>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>Sonicated homogenates</td>
<td>155 ± 6</td>
<td>12.2 ± 0.9</td>
<td>64 ± 3</td>
<td>320 ± 10</td>
</tr>
<tr>
<td>(98 ± 3%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>Sum for the 10 fractions</td>
<td>142 ± 6</td>
<td>11.6 ± 0.9</td>
<td>62 ± 2</td>
<td>300 ± 10</td>
</tr>
<tr>
<td>(91 ± 3%)</td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
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</tr>
</tbody>
</table>
the earlier studies (Kuzma et al., 1999) and was probably due to a combination of modifications. Homogenization and fractionation of only the dissected CZ tissue instead of whole nodules avoided contamination and by maintaining the frozen material at about \(-15^\circ C\) during lyophilization avoided any minor redistribution of solutes. The 10 fractions collected from the gradient were immediately divided into aliquots for assay, rather than pelleting and resuspending in heptane as previously (Kuzma et al., 1999), thereby reducing losses.

The detection limit for nonaqueous fractionation of a metabolite in a particular compartment has been estimated at about 5% of the total amount in intact cells (Farre et al., 2001). The pool of total adenylates in mitochondria was estimated to be \(10^6\)–2% of the total adenylate amounts in the infected cells (Fig. 4), a value that may be considered reliable. Nevertheless, variation in the measured adenylate contents found for mitochondria was slightly greater than that in the plant cytosol and bacteroids (Fig. 4).

Separation of subcellular compartments using this method was unable to resolve plastids, which would undoubtedly have contained some adenylates. The Bestfit program that was used (Riens et al., 1991) assumed the metabolites recovered were only localized in three compartments (bacteroids, plant cytosol, and mitochondria). Although the amount of adenylate in plastids may not be significant it would have been included in one or all of the compartments defined above. Given the likely density of plastids, their contents would be more likely to add to the mitochondrial and bacteroid compartments rather than the cytosol.

### Effect of Ar:O₂ on Adenylate Pools in Subcellular Compartments of Nodules

A mathematical model developed previously (Wei et al., 2004) predicted that a large sink for ATP in the cytosol compartment was responsible for generating an adenylate gradient in the plant fraction of the legume nodule. The model also predicted that Ar:O₂ would reduce the ATP demand for \(NH_4^+\) assimilation in the plant cytosol, causing the average AEC value in plant cytosol to increase significantly within a few minutes of Ar:O₂ exposure. The results of this study provided support for this prediction; the AEC of plant cytosol increasing, from \(0.63 \pm 0.02\) in \(N_2:O_2\) to \(0.73 \pm 0.02\), within 7 min of Ar:O₂ exposure (Fig. 5A). While the experimental data cannot address the issue of significant gradients in AEC across the infected nodule cell, the response to short term Ar:O₂ exposure, such that the AEC of the mitochondrial compartment remained constant while that of the bacteroids became progressively more typical of severe O₂ deprivation, is consistent with the existence of such a gradient.

Furthermore, the significant increase in the AEC of plant cytosol observed following Ar:O₂ exposure

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**Figure 4.** Effects of Ar:O₂ exposure on the pool sizes of ATP (●), ADP (▲), and AMP (●) in the bacteroid (A), plant cytosol (B), and mitochondria (C) compartments in CZ tissue from nodules of 4- to 5-week-old soybean plants. Values are presented as the mean (±SE; \(n = 3\)) for each of the three treatments (\(N_2:O_2\), 7-min, and 60-min Ar:O₂ exposure).

**Figure 5.** Effect of Ar:O₂ exposure on the AEC (A) and ATP-ADP ratio (B) in the bacteroid (▲), plant cytosol (□), and mitochondria (○) compartments of the CZ tissue from nodules of 4- to 5-week-old soybean plants. Values are presented as the mean (±SE; \(n = 3\)) for each of the three treatments (\(N_2:O_2\), 7-min, and 60-min Ar:O₂ exposure).
supports the hypothesis being tested in this study and is consistent with a reduced demand for ATP in the cytosol where, in Ar:O\textsubscript{2}, NH\textsubscript{4}\textsuperscript{+} assimilation through GS activity would be negligible. Since nodule respiration (and presumably ATP synthesis) after 7 min Ar:O\textsubscript{2} exposure is similar to that in air (King and Layzell, 1991), the decrease in cytosolic ATP consumption would result in a rise in AEC and ATP-ADP ratio in the plant cytosol under the Ar:O\textsubscript{2} treatment. The fact that Ar:O\textsubscript{2} exposure reduces nodule respiration after 60 min exposure (King and Layzell, 1991), yet the AEC and ATP-ADP ratio continues to rise, provides evidence that the decline in ATP demand outstrips any decrease in the rate of mitochondrial ATP synthesis during the Ar-induced decline.

Within the first 7 min of Ar:O\textsubscript{2} exposure, the bacteroid AEC was unchanged from the value in air (0.73 ± 0.02), but declined sharply (to 0.57 ± 0.03) after 60 min Ar:O\textsubscript{2} exposure (Fig. 5A). Previous studies have shown that long-term exposure of legume nodules to an Ar:O\textsubscript{2} atmosphere causes a major reduction in the permeability of the nodule to O\textsubscript{2} diffusion, thereby reducing the infected cell O\textsubscript{2} concentration (King and Layzell, 1991) and imposing a severe O\textsubscript{2} limitation on nodule metabolism.

Kuzma et al. (1999) employed a nonaqueous fractionation technique, similar to that used in this study, to show that a low O\textsubscript{2} treatment of nodules decreases the AEC of bacteroids while having little or no effect on the cytosolic AEC. This finding showed that the bacteroids were the site of O\textsubscript{2} limitation in nodules. Since similar results were obtained in this study, our findings were fully consistent with the 60-min Ar:O\textsubscript{2} exposure imposing an O\textsubscript{2} limitation on nodule metabolism that impacted bacteroid metabolism, even as the plant cytosolic fraction had a less severe O\textsubscript{2} limitation, as reflected by an increase in AEC.

The average mitochondrial AEC in the CZ of soybean nodules was high before and after Ar:O\textsubscript{2} treatment. This result was consistent with the bacteroid, rather than the plant compartment, being the site of O\textsubscript{2} limitation in legume nodules (Kuzma et al., 1999), even in nodules that were severely O\textsubscript{2} limited from low O\textsubscript{2} treatment (Kuzma et al., 1999) or from Ar:O\textsubscript{2} exposure (King and Layzell, 1991). Maintenance of O\textsubscript{2} sufficiency in mitochondria of cells in which the bacteroids are O\textsubscript{2} limited can be explained by the localization of mitochondria at the periphery of the infected cells, adjacent to intercellular gas spaces. Models of O\textsubscript{2} diffusion into the infected cells have predicted the presence of very steep gradients in O\textsubscript{2} concentration from the intercellular spaces to the center of the large, infected cells (Bergersen, 1994; Thumfort et al., 1994, 1999, 2000). On a whole-nodule basis, there was little effect of extended Ar:O\textsubscript{2} treatment on the summed or measured AEC, despite the fact that other treatments known to induce an O\textsubscript{2} limitation of nodule metabolism (low O\textsubscript{2}, nitrate fertilization, stem girdling) have been shown to result in a significant decrease in the nodule AEC (de Lima et al., 1994). However, these other treatments may affect only the O\textsubscript{2} status of the nodule, and have an indirect effect on the demand of GS for cytosolic ATP. On the other hand, the Ar:O\textsubscript{2} treatment both alters the O\textsubscript{2} status of the nodule through increased diffusion resistance and eliminates adenylate demand for NH\textsubscript{4}\textsuperscript{+} assimilation. Therefore, the decreasing AEC in the bacteroid compartment following Ar:O\textsubscript{2} exposure was offset by a concomitant increase in cytosolic AEC caused by a reduction in the ATP demand for GS activity. This could explain the lack of a decline in whole-nodule AEC following Ar:O\textsubscript{2} treatment (1 h) observed in an earlier study (de Lima et al., 1994).

Adenylates and Regulation of Glycolytic Flux in Ar-Exposed Nodules

Curioni et al. (1999) found direct evidence linking N deprivation to glycolytic flux in alfalfa (Medicago sativa) nodules exposed to Ar:O\textsubscript{2}. The Glu-Gln ratio increased 5-fold during the first 30 min, while malate concentration declined and Suc accumulated. The study also showed ratios of Fru-6-phosphate to Fru-1,6-bisphosphate and phosphoenolpyruvate to pyruvate to increase after 30 min Ar-exposure, consistent with a negative effect of N deprivation on the activity of phosphofructokinase (PFK) and pyruvate kinase. Although it was proposed that the increased Glu-Gln ratio, caused by NH\textsubscript{4}\textsuperscript{+} unavailability, may act as a signal to decrease plant glycolytic flux (Curioni et al., 1999), a role for adenylates, and specifically elevated ATP, can be envisaged. Since the turnover of ATP and ADP is coupled to several steps in both NH\textsubscript{4}\textsuperscript{+} assimilation and glycolysis, we propose that the observed increase in cytosolic ATP-ADP ratio in Ar (this study) will inhibit the activity of some key glycolytic enzymes, resulting in a reduced C-flux through the pathway.

Previous studies (Larsson et al., 2000) showing that ATP has a strong negative effect on glycolysis in yeast (Saccharomyces cerevisiae) by affecting several glycolytic enzymes, including PFK, pyruvate kinase, hexokinase, and enolase, support this idea. In fact, in chickpea (Cicer arietinum) nodules cytosolic PFK was disaggregated in the presence of ATP resulting in a lower affinity for its substrate, Fru-6-phosphate (Lee and Copeland, 1996). Another key enzyme in nodule C metabolism is PEPC, which is regulated at the post-translational level in soybean, alfalfa, and cowpea (Vigna unguiculata) nodules (Pathirana et al., 1992; Schuller and Werner, 1993; Vance et al., 1994; Smith et al., 2002) by protein phosphorylation. PEPC activity became more sensitive to inhibition by maltose after a few hours in Ar (Smith et al., 2002), and it is conceivable that changes in adenylate status would affect its phosphorylation, thereby contributing to a reduced flux of C through glycolysis.

The simulation model (Figure 5A in Wei et al., 2004) predicted a substantial gradient in ATP concentra-
tion (1.62–1.04 mM in ureide-exporting nodules and 1.62–0.79 mM in amide-exporting nodules) across the cytosol of the infected plant cell. If this indeed occurs, then it is likely that a gradient exists in the amount of glycolytic regulation across the cells, thereby creating the potential for very dynamic and complex interactions in the cytosol.

Are Adenylates Signal Molecules for the Control of Nodule Oxygen Permeability?

While there is abundant physiological evidence that a reversible mechanism controlling the permeability of nodules to gaseous diffusion has a central regulating role in determining rates of nitrogenase activity, the nature of the signals involved and the transduction pathway linking this mechanism to metabolic events in the nodule remains obscure (for review, see Minchin, 1997).

The rapid and significant adjustment in plant cytosolic AEC and ATP-ADP ratio that accompanies the reduced flux of fixed N to the plant cell cytosol on exposure of nodules to 7 min Ar:O₂ (80:20; v/v) raises the possibility that adenylates may, in fact, be signal molecules for the control of nodule O₂ permeability. This might be mediated through activation of ion pumps (i.e. ATP-dependent K⁺ - and/or Ca²⁺ -pumps) or ATP-gated ion channels on the plasma membrane that results in a net efflux of K⁺ and Ca²⁺ from the cytoplasm of the infected cells to the intercellular spaces. The K⁺, Ca²⁺, and water in the intercellular spaces of the CZ region would be likely to move toward the inner cortex region because the intercellular spaces in the inner cortex are smaller and therefore have stronger hydrogen bonding between water molecules and the cell walls (i.e. capillary effect). Such a relocation of ions and water, from the intercellular space in CZ to that in the inner cortex, would decrease nodule permeability to O₂ and reduce O₂ availability to infected cells. That could account for the greater O₂ limitation on nitrogenase activity and bacteroid metabolism that is known to be associated with Ar:O₂ exposure (King and Layzell, 1991).

Minchin et al. (1995) have provided some preliminary evidence for increases in the K⁺ and Ca²⁺ contents of inner cortical tissue of soybean nodules following treatments that increased diffusive resistance. However, studies linking intra- and intercellular ion fluxes to metabolic events in infected cells of the central tissue zone have yet to be undertaken. The hypothesis that AEC and/or ATP levels provide this link is now being tested in the laboratory of the corresponding author.

A recent report has speculated that ATP might have a role in plants as an extracellular signal molecule, functioning in a manner similar to its role as a neurotransmitter in animal systems (Demidchik et al., 2003). While the sources and status of extracellular adenylates and their possible interaction with plasma membrane purinoceptors in plants are not yet clear, such a mechanism is envisaged to adjust the level of cytosolic Ca²⁺ via activation of Ca²⁺-permeable membrane channels (Demidchik et al., 2003). If this external pool of adenylates reflected AEC within the infected cells, then perhaps it is not so unlikely for intracellular ATP to function as a regulator of gaseous permeability in nodules through an extracellular ATP pool.

MATERIALS AND METHODS

Plant Culture

Seeds of soybean (Glycine max L. Merr. cv Maple Arrow) were inoculated at the time of planting with Bradyrhizobium japonicum USDA 16 at about 0.5 × 10⁹ viable cells per plant. The seeds were planted 3 cm deep under the surface of silica sand (grade 16) in a gas-exchange pot and grown in a growth chamber (Kuzma and Layzell, 1994). For the first 2 weeks after sowing, plants were irrigated twice daily with a nutrient solution containing 0.5 mM KNO₃. Subsequently, plants were watered with N-free nutrient solution. After 4 to 5 weeks of growth, nitrogenase activity was measured and nodule tissue prepared for fractionation and adenylate assays.

N₂O₃ and Ar:O₂ Treatment and Nodule Harvesting

Three uniform populations of 18 soybean plants were used. Within a population, six plants were selected for measurement of ANA (Hunt et al., 1989) in N₂O₃ (80:20; v/v) treatment and then immediately harvested. Another six plants were first assayed for ANA, then exposed to 5 to 7 min of Ar:O₂ (80:20; v/v) treatment to obtain a measurement of TNA (Hunt et al., 1989) and then harvested. The last six plants were assayed for ANA, then exposed to 60 min of Ar:O₂ treatment to obtain a second measurement of TNA and then harvested.

The nodules were harvested by quickly uprooting the soybean plant and immediately immersing the roots with rooting medium attached in liquid N₂ (−196°C). The nodules were picked from the frozen roots and solid matrix while being kept frozen by occasional treatment with liquid N₂, weighed, and stored in liquid N₂. Specific ANA and/or TNA values for each plant were calculated, taking care to include the weight of nodule tissue that was missed in the initial harvest (typically 10%–20% of total). The ANA values of the nodules from all plants except one for the 60-min Ar:O₂ treatment were in the range of 70 to 100 µmol H₂ g⁻¹ DW(nod) h⁻¹ (assuming a ratio of 4.8 for fresh to dry weight); therefore, one plant for the 60-min Ar:O₂ treatment was discarded.

Nodule Lyophilization and Dissection

The soybean nodules from the same treatment within the same population were mixed. Therefore, the three plant populations now became nine samples of mixed nodules (i.e. 3 treatments × 3 replicates per treatment). To facilitate lyophilization, frozen large nodules (>2 mm diameter) were cut in half while immersed in liquid N₂ using a small guillotine fabricated from razor blades. For each of the nine samples, two 100-mg aliquots of nodule halves were set aside for metabolite and marker enzyme assay. The remaining nodule material was lyophilized in Fast-Freeze Flasks (Labconco, Kansas City, MO) while being maintained at −15°C in a modified chest freezer for the first 2 d, and at room temperature for another 2 d. The cold trap temperature was set at −50°C and the vacuum at 6 to 8 microns (18 L Freeze Dry System, Labconco).

To provide homogenous CZ tissue for nonaqueous fractionation, lyophilized nodule halves were dissected into cortex and CZ tissues using a dissecting needle and microforces. This was done in a partially closed chamber under a dissection microscope while the chamber was flushed continuously with a stream of compressed air to maintain a dry environment. The cortex and CZ tissues have different textures and water contents (Oresnik and Layzell, 1994), and as a consequence were easily separated. Dissected, dry cortex and CZ tissues were weighed using a microbalance (Cahn C-31) and then stored in a tightly sealed tube inside a desiccator maintained at −20°C.
Nonaqueous Fractionation of Central Zone Tissue

The dried CZ tissue was ground in a mortar and pestle in a gas bag filled with dry N₂ gas and nonaqueous fractionation carried out using a modified method of Kuzma et al. (1999). An aliquot (250–300 mg) was suspended in 20 mL loading solvent (heptane/tetrachloroethylene mixture with a density of 1.23 g mL⁻¹), and sonicated (model 450 sonicator with a stepped microprobe, Branson Ultrasonics, Danbury, CT) at a power output of 3 and a duty cycle of 33% for 8 min, and then at an output of 3 and duty cycle of 12% for 4 min. The sonicated homogenate was filtered through 82-μm nylon mesh, pelleted, and resuspended in 4.5 mL loading solvent. Two 0.25-mL aliquots were set aside for metabolite and marker enzyme assays, and the remaining 4 mL loaded onto the top of a nonaqueous gradient having a density range of 1.23 to 1.40 g mL⁻¹ (Kuzma et al., 1999).

Three gradients were prepared simultaneously, one for each of the treatment groups from a plant population. The gradient was centrifuged at 25,000g for 2.5h (rotor SW28, Beckman 18-55M, Beckman Instruments, Fullerton, CA) and 10 fractions collected from each as described previously (Kuzma et al., 1999). Each fraction was divided into two, one half for adenylate assay and the other for marker enzyme and protein analysis. Each subfraction was then diluted 3-fold with heptane, centrifuged (18,000g, 8 min) and the pellet dried under vacuum. The dried subfractions were held in Eppendorf tubes inside a desiccator maintained at –20°C.

Tissue Extraction and Analysis of Adenylate, Protein, and Marker Enzymes

Adenylates and marker enzymes were assayed in extracts of frozen nodules, dry cortex and CZ tissues, and in dry subfractions as described previously (Kuzma et al., 1999). Protein content was determined using a Protein Assay ESL Kit (Böhringer Mannheim).

To assess the stability of adenylates during extraction in 10% perchloric acid, a known amount of ATP, ADP, and AMP (10 or 20 nmol) was added to an aliquot of the fresh nodule tissues and also to the dried cortex and CZ tissue samples. After correction for endogenous adenylate content, recovery of ATP, ADP, and AMP was 91 ± 3%, 92 ± 5%, and 95 ± 6% (n = 9), respectively, and no differences were found between the three nodule tissues. Given the consistently high recovery rate, no adjustments were made to measured adenylate levels in subfractions. Recovery of adenylate pools, protein, and marker enzyme activities were also calculated by comparing the measured levels in dried CZ with those from all subfractions.

Determination of Adenylate Contents in the Plant Cytosol, Mitochondria, and Bacteroid Fractions

The proportion of each of the adenylates, soluble protein content, and marker enzyme levels in each gradient were entered into a computer program (Bestfit) developed by Rens et al. (1991). As described previously (Kuzma et al., 1999; Farre et al., 2003), this program used a deconvolution approach to reconstruct the adenylate and protein pools in each of the three compartments represented by marker enzymes: GDH for mitochondria, PEPC for plant cytosol, and HBD for bacteroids. The adenylate contents in the individual compartments of all 10 fractions of the gradient were summed so that the adenylate pools in the individual compartments of all 10 fractions of the gradient could be constructed.

Total soluble protein was treated as a metabolite. Thus, the distribution of protein among the three compartments could also be calculated using the Bestfit program to analyze the measured contents of protein and the activities of marker enzymes in each fraction of the gradient.

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LITERATURE CITED


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