Effect of proline on nitrogenase activity in symbiosomes from root nodules of soybean (Glycine max L.) subjected to drought stress

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Received 20 February 1996; Accepted 18 June 1996

Abstract

Experiments were carried out to investigate if drought stress affects the ability of bacteroids from soybean (Glycine max L.) root nodules to utilize proline and malate to support nitrogenase activity. The bacteroids were isolated in sub-ambient oxygen and nitrogenase activity was measured by acetylene reduction. Nitrogenase activity supported by proline was 8-fold higher in bacteroids from drought-stressed nodules than in bacteroids from control nodules. In contrast to the results with proline there was no significant response to drought stress in the rate of bacteroid nitrogenase activity supported by malate. The effect of drought stress on transport of proline and malate across the symbiosome membrane was investigated by incubation of symbiosomes isolated in sub-ambient oxygen with radioactive tracers. Drought stress tended to increase the rate of proline uptake relative to a minor decrease in malate uptake into symbiosomes in response to drought. There was no indication of a saturable carrier in the symbiosome membrane for either substrate at concentrations in the range 0.1–2 mM. The rate of malate uptake into symbiosomes was twice as high as the rate of proline uptake at all substrate levels tested. The protein composition of the symbiosome membrane was altered in response to drought stress and these changes may relate to the permeability of the symbiosome membrane.

Key words: Drought stress, nitrogenase activity, proline, soybean nodules, symbiosome membrane, transport.

Introduction

The metabolic potential of legume root nodules is reduced during drought (Díaz del Castillo et al., 1994; Díaz del Castillo and Layzell, 1995) and even mild drought results in a decline in activity and in content of sucrose synthase which is involved in sucrose metabolism in legume nodules (González et al., 1995). These changes may influence the range of carbon substrates available to the bacteroids during drought periods. Several investigations have demonstrated that dicarboxylates are the main energy-yielding substrates supplied to the bacteroids under non-stressed conditions (Reibach and Streeter, 1984; Udvardi et al., 1988a; Tajima and Kouchi, 1990), but carrier-mediated transport across the bacteroid membrane (BM) has been reported also for fructose (Udvardi et al., 1990) and amino acids (Streeter and Salminen, 1988; Udvardi et al., 1988b). It is, however, transport across the plant-derived membrane surrounding the bacteroids (the symbiosome membrane, SM), that determines which compounds the bacteroids have access to within the nodule. Transport studies with isolated symbiosomes (bacteroids surrounded by SM) have demonstrated a dicarboxylate carrier in SM that mediates rapid transport of dicarboxylates such as malate and succinate (Udvardi et al., 1988a; Herrada et al., 1989; Ou Yang et al., 1990).

Drought stress stimulates an accumulation of proline in plant tissue which results in a 10–100-fold increase in the amount of proline in leaves (Aspinall and Paleg, 1981) and a 4–5-fold increase in the content of proline in legume root nodules (Kohl et al., 1991). The lower proline accumulation in nodules compared to leaves may be a result of the higher water potential in nodules compared to leaves at any level of total plant drought.
stress (Huang et al., 1975). Proline is believed to act as an osmoticum in plant cells (Kauss, 1977; Hu et al., 1992; Dellauney and Verma, 1993). Proline may serve as a stabilizing agent for membranes (Rudolph et al., 1986) and van Rensburg et al. (1993) have shown that there is a positive correlation between proline content of tobacco leaves and membrane integrity, measured as ion leakage. The molecular mechanism underlying this effect of proline is not completely understood but Rudolph et al. (1986) have suggested that proline may affect the hydration layer surrounding phospholipids.

Aside from accumulation of proline in nodules, drought stress also induces an increased activity of enzymes related to proline metabolism in bacteroids (Kohl et al., 1991). This suggests that proline may be imported to the symbiosomes as a substrate for bacteroids during periods of drought stress. Results by Udvardi et al. (1990) demonstrate a slow uptake rate and no evidence for carrier-mediated transport of proline into symbiosomes isolated aerobically from soybean nodules that were not drought stressed. Thus, a change in the permeability of SM in response to drought stress is necessary for proline to play a significant role in bacteroid metabolism. Drought stress may change the permeability of SM, since drought stress affects membrane stability (Liljenberg, 1992; van Rensburg et al., 1993).

It can not be excluded that transport properties of SM in vivo where symbiosomes are maintained at sub-ambient oxygen differ from those reflected by in vitro experiments with symbiosomes isolated at ambient air. Nitrogenase activity is inhibited by oxygen tension of ambient air and symbiosomes devoid of nitrogenase activity are likely to differ from symbiosomes with an active nitrogenase with respect to the internal molecular composition. This may affect transport properties of the SM. Furthermore, oxygen induces membrane depolarization in legume nodules (Denison and Kinraide, 1995). The underlying biochemical mechanism has not been identified, but oxygen-induced membrane depolarization may also apply to SM when isolated at ambient air and thus affect SM transport properties.

The aim of this work was to investigate the effect of drought stress on the ability of bacteroids to utilize proline to support nitrogenase activity. It was investigated if drought stress has an effect on the passage of proline into isolated symbiosomes. The experiments were carried out using symbiosomes and bacteroids isolated at sub-ambient oxygen and oxygenated leghaemoglobin was added during incubations. In this way bacteroids maintain nitrogenase activity and a possible membrane depolarization in response to oxygen is avoided. Parallel experiments with malate as a substrate were performed as malate is believed to be an important substrate for the bacteroids under non-stressed conditions. The effect of drought stress on SM was further examined by investigation of the polypeptide profile of SM from control and drought-stressed nodules.

Materials and methods

L-[U-14C] proline (9.47 GBq mmol⁻¹) and L-[U-14C] malic acid (1.90 GBq mmol⁻¹) were purchased from Amersham Life Science, Amersham, UK. Silicone-oil (AR-200) was obtained from Wacker Chemie, München Werk Burghausen, Germany. Scintillation liquid was purchased from Packard Instr., Groningen, The Netherlands. All other chemicals were purchased from Sigma. Oxygenated soybean leghaemoglobin was purified as described by Appleby and Bergeres (1980).

Plant growth

Soybean (Glycine max (L.) Merr. cv. Evans) seeds were surface-sterilized, sown in vermiculite, and inoculated at sowing with a 3-d-old yeast broth suspension culture of Bradyrhizobium japonicum strain 110 (Hahn and Hennecke, 1984). Pots with four plants were grown in a modified Leonard jar system (Rosendahl and Jakobsen, 1987) in a growth cabinet with an average daily irradiance of 470 μmol m⁻² s⁻¹ (PAR). The temperature during the 16/8 h day/night cycle was 20/16°C. The plants were supplied with a nutrient solution (Rosendahl and Jakobsen, 1987) containing 6 mM Ca(NO₃)₂ at the time of sowing and later nitrogen-free nutrient solution was added until harvest at the age of 6 weeks.

Drought stress induction and its reflection in acetylene reduction rate of nodulated root systems

To attain different levels of drought stress the nutrient-solution containers were drained in duplicate and the plants were left to dry for 40, 56 and 80 h prior to recording of nitrogenase activity by acetylene reduction assays. For each drought stress period, one pot of four plants was assayed and the other rewatered and assayed 24 h later. The acetylene reduction rate of the whole root system of each plant was assayed separately in a 550 ml rubber capped glass bottle. 10% (v/v) acetylene was added and ethylene production was measured on 0.5 ml headspace samples collected after 10, 20, 30, and 40 min using a Hewlett Packard 5730A gas chromatograph equipped with a flame ionization detector and a Porapak T (80–100 mesh) column.

Plants subjected to 40 h drought stress were chosen for all further experiments. At this level of drought stress the plants were visibly starting to wilt and the rate of acetylene reduction was decreased by 60% compared to control plants, but 24 h after rewatering acetylene reduction was recovered (Fig. 1).

Preparation of symbiosomes and bacteroids

Twenty grams of nodules from control plants and from plants subjected to 40 h drought stress were harvested and placed on ice. All further handling of the nodules was performed at 4°C at sub-ambient oxygen (N₂ gas) in an AtmosBag (Aldrich) and buffers were chilled and purged with N₂ prior to use. Symbiosomes were isolated by Percoll density-gradient centrifugation as previously described for pea-Rhizobium symbioses (Rosendahl et al., 1992) except for the use of a Percoll concentration of 50% in the continuous Percoll gradients. The change in the Percoll concentration was required because soybean symbiosomes have a higher density than pea symbiosomes. The integrity of the symbiosomes was verified by microscopy with a Zeiss light microscope equipped with...
35x116](0.25 mm diameter) was introduced into each Eppendorf tube. The mixture could then be removed by pulling the iron thread. The layer of reaction mixture was immersed into liquid N for a few seconds. The layer of reaction mixture was removed while the acid layer was kept frozen. The acid layer containing the symbiosomes was transferred to a scintillation vial and the radioactivity was measured on a liquid scintillation analyser (Packard 1900 TR). All reactions were conducted in triplicate at 20°C.

**SDS-gel electrophoresis**

Symbiosome membranes from drought-stressed and control nodules were prepared by whirly mixing symbiosome suspensions 2–3 min in wash buffer. The bacteroids were removed by centrifuging at 4000 g for 15 min. The supernatant was ultracentrifuged at 100 000 g for 30 min to pellet SM. The SM pellets were solubilized in a buffer containing 60 mM Tris (pH 6.8), 2% SDS, 5% mercaptoethanol, and 10% glycerol.

SDS-PAGE (10%) was performed as described by Laemmli (1970). Each lane was loaded with 20 μg SM protein. The protein bands were visualized by silver staining.

**Results**

**Nitrogenase activity**

Drought stress resulted in a severe decrease in nitrogenase (acetylene reduction) activity of nodulated root systems (Fig. 1). The present data on acetylene reduction of root systems were obtained using the conventional method on detopped systems in closed vessels. This method does not apply for recordings of accurate values of nitrogenase activity (Minchin et al., 1986). However, in the present work the acetylene reduction assay on root systems was performed merely to establish the degree of drought stress that affects nodule performance in a reversible manner and for this purpose the conventional method of acetylene reduction is suitable. A 60% decrease in nitrogenase activity resulted from 40 h drought. Prolonging the drought treatment to 56 h did not result in a further decrease whereas 80 h drought resulted in an 80% decrease in nitrogenase activity. Nitrogenase activity was completely recovered 24 h after rewatering the 40 h drought-stressed plants whereas the ability for immediate recovery of nitrogenase activity was lost at drought treatments exceeding 40 h (Fig. 1). All further experiments with drought stress were performed with plants subjected to the reversible 40 h drought treatment.

The ability of proline to support nitrogenase activity in drought-stressed nodules was examined on bacteroids isolated at sub-ambient oxygen from drought-stressed...
and control nodules. As isolated bacteroids were used, the results provide a direct measurement of the capacity of the substrate to support nitrogenase activity. Incubation of bacteroids in wash buffer with no additional substrate resulted in nitrogenase activity at the detection limit (Fig. 2). This excludes the possibility of nitrogenase activity supported by mannitol present in the wash buffer. Supplementing the incubation medium with proline (5 mM) increased nitrogenase activity in bacteroids from control as well as drought-stressed nodules. The level of nitrogenase activity supported by proline was 8-fold higher in bacteroids from drought-stressed nodules than in bacteroids from control nodules (Fig. 2).

Malate supplied at the same concentrations as proline is superior to proline in support of acetylene reduction in bacteroids isolated from control as well as drought-stressed nodules (Fig. 2), however, in contrast to the results with proline there was no significant change in the rate of nitrogenase activity supported by malate in response to drought stress.

Transport studies

The effect of drought stress on the rate of substrate uptake into symbiosomes was investigated by incubating symbiosomes isolated at sub-ambient oxygen with different concentrations of $^{14}$C-proline or $^{14}$C-malate. Drought stress tended to increase the rate of proline uptake into symbiosomes relative to a minor decrease in the rate of malate uptake in response to drought stress (Fig. 3). This could indicate a change in the transport properties of the SM, but it may well result from an increased rate of proline metabolism in the bacteroids from drought-stressed nodules. The rate of proline and malate uptake increased linearly at increasing substrate concentrations, thus there is no indication of a saturable carrier for either substrate in SM at the investigated substrate concentrations. The level of proline uptake in these symbiosomes isolated at sub-ambient oxygen is 3-fold higher than previously reported for soybean symbiosomes isolated and incubated under aerobic conditions (Udvardi et al., 1990). In general, the rate of malate uptake is about twice as high as the rate of proline uptake, independent of the drought stress treatment and the level of malate uptake in the present study corresponds to levels reported in other work (Udvardi et al., 1990).

Effect of drought stress on SM

Drought stress resulted in a decrease in the density of symbiosomes. Symbiosomes from control plants form a dense band at approximately $\rho$ 1.107 in the Percoll gradients, whereas symbiosomes from drought-stressed plants form a more diffuse band in the density range $\rho$ 1.096 to 1.107 (data not shown).

The protein composition of SM was affected by drought stress as evident from a silver stain of an SDS-PAGE (Fig. 4). The majority of polypeptide bands were fainter or missing in SM from drought-stressed plants compared to SM from control plants and few polypeptide bands retained or increased the intensity in response to drought stress (Fig. 4, arrows). The lanes of the gel in Fig. 4 were
The matrix of spinach leaf mitochondria has been shown to be biphasic; at concentrations higher than 0.5 mM an active uptake system becomes apparent (Yu et al., 1983). The present results on uptake of proline into symbiosomes (Fig. 3) reflect uptake by diffusion, since there is no indication of saturation for the concentrations investigated (0.1, 0.5, 1, and 2 mM). This is consistent with data reported by Udvardi et al. (1990) based on symbiosomes incubated with 5 μM to 2 mM proline. The proline concentrations used in the experiments are physiologically relevant as the concentration of proline in roots of legume nodules is approximately 1 mM. The proline concentration of control nodules is about 4 times lower (Kohl et al., 1991).

The rate of proline uptake in the present study is approximately three times higher than that reported by Udvardi et al. (1990). This discrepancy may be because symbiosomes and bacteroids were isolated in sub-ambient oxygen that maintain nitrogenase activity. This results in a different internal molecular composition of symbiosomes compared to symbiosomes devoid of nitrogenase activity and it may result in differences in symbosome sink strength for various substrates. Furthermore, oxygen induces rapid membrane depolarization in legume nodules (Denison and Kinraide, 1995). Provided the effect of oxygen on membrane polarization also applies to SM when isolated at ambient air this may contribute to the observed differences in SM transport properties between the present results and results obtained with symbiosomes isolated and incubated at ambient air (Ou Yang et al., 1990; Udvardi et al., 1990).

Drought stress reduces the level of total soluble protein in legume nodules (Gogorcena et al., 1995). The alterations in the protein composition of SM as a result of drought stress (Fig. 4) compare to alterations in the protein composition of SM as a response to induced senescence found by Jacobi et al. (1994). Induced senescence in soybean root nodules by plant decapitation caused extensive degradation of some nodule specific proteins (nodulins) in SM whereas ageing did not have the same effect (Jacobi et al., 1994). The response of the induced senescence may be a general stress response and it is, therefore, likely that the alterations in the polypeptide pattern in response to drought stress, observed in this investigation, reflect degradation of several specific SM proteins. Changes in the protein composition of SM in response to drought (Fig. 4) may relate to transport properties of SM causing the observed changes in the rate of proline and malate uptake into symbiosomes (Fig. 3).

The uptake of proline into symbiosomes is likely to be mediated by diffusion, therefore, the uptake will be dependent on the concentration of proline in the incubation media. The uptake of proline into symbiosomes (Fig. 3) reflect uptake by diffusion, since there is no indication of saturation for the concentrations investigated (0.1, 0.5, 1, and 2 mM). This is consistent with data reported by Udvardi et al. (1990) based on symbiosomes incubated with 5 μM to 2 mM proline. The proline concentrations used in the experiments are physiologically relevant as the concentration of proline in roots of legume nodules is approximately 1 mM. The proline concentration of control nodules is about 4 times lower (Kohl et al., 1991).

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tion mixture as well as the ability of the bacteroids to utilize proline. Proline uptake into symbiosomes from drought-stressed nodules is slightly increased compared to the uptake into symbiosomes from control nodules (Fig. 3). The increased potential for proline metabolism in bacteroids from drought-stressed nodules reflected by increase in ProDH activity (Kohl et al., 1991) may not be met by the increase in proline uptake. However, the recorded rate of proline uptake into symbiosomes from drought-stressed nodules may underestimate the actual uptake as a larger proportion of the \(^{14}\text{C}\)-proline transported into symbiosomes may be metabolized and \(^{14}\text{C}\) lost as \(^{14}\text{CO}_2\) as a result of increased capacity for proline metabolism in the bacteroids. This assumption is based on the finding by Salminen and Streeter (1990) that, despite short incubation times, substrate uptake estimates based on incubation experiments may underestimate the actual uptake because substrate is metabolized during the incubation and radioactivity thereby lost as \(^{14}\text{CO}_2\).

The role of proline in supporting nitrogenase activity during drought stress may depend on the availability of other substrates, primarily organic acids, and also on the uptake rate of these substrates into symbiosomes during drought stress. A rate of proline uptake of 12 nmol min\(^{-1}\) mg\(^{-1}\) protein (Fig. 3; 2 mM external proline concentration) into symbiosomes from drought-stressed nodules may support some nitrogenase activity during periods of drought stress. However, the increased concentration of proline in the root nodules may have other effects as well. There is a positive correlation between proline content of tobacco leaves and membrane integrity (van Rensburg et al., 1993) and proline is believed to stabilize membrane phospholipids (Rudolph et al., 1986). Thus a general stabilizing effect of proline on membranes including SM may represent an additional effect of proline increasing the ability of the symbiosomes to overcome periods of drought stress.

Acknowledgements

We wish to thank Dr Daniel Kohl who inspired us to pursue the subject, Ina Hansen for excellent technical assistance, and Dr Sören Christensen for critical reading of the manuscript. The research was financially supported by the Danish Agricultural and Veterinary Research Council, Grant no. 23–2700.

References


