RESPIRATION AND NITROGEN FIXATION BY BACTEROIDS FROM SOYBEAN ROOT NODULES

SUBSTRATE TRANSPORT AND METABOLISM IN RELATION TO INTRACELLULAR CONDITIONS

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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DECLARATION

The research presented in this thesis is my own work unless otherwise stated elsewhere. The project was undertaken in the School of Biochemistry and Molecular Biology, Faculty of Science, at the Australian National University. The material presented in this thesis has not been submitted for any other degree.

Part of work on the bacteroid TCA cycle was completed by Dr Laura Green who was a post-doctoral fellow in Professor David A. Day's research group.

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Dear Dad, this is for you from your son

ABRREVIATIONS

ABC	ATP binding cassette
ADH	Alanine dehydrogenase
AMT	Ammonia transport family
BNF	Biological nitrogen fixation
Bra	Branched-chain amino acid transporter
cMDH	cytosolic MDH
cDNA	copyDNA
Da	Dalton
Dct	Dicarboxylate transport system
DTT	Dithiothreitol
DW	Dry weight
EDTA	Ethylenediaminetetraactetic acid
GOGAT	Glutamine-oxoglutarate amino transferase
GS	Glutamine synthetase
KDa	Kilo Daltons
Lb	Leghaemoglobin
LbO_2	Oxy-leghaemoglobin
MES	2-[N-Morpholino]ethanesulphonic acid
ME	Malic enzyme
MDH	Malate dehydrogenase
MOPS	3-[N-Morpholino]ethanesulphonic acid
NAD(H)	Nicotine-adenine dinucleotide (reduced)
NADP(H)	Nicotine-adenine dinucleotide phosphate (reduced)
neMDH	nodule-enhanced MDH
nmol	nanomole
PBM	Peribacteroid membrane
PBS	Peribacteroid space
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PHB	Poly-β-hydroxybutyrate
PVP	Polyvinyl pyrrolidone
RB	Reaction buffer
SS	Sucrose synthase
VHose	Vacuum connection hose
v/v	volume/volume
w/v	weight/volume
YMA	Yeast extract-mannitol agar medium
YMB	Yeast extract-mannitol medium

ABSTRACT

Bacteroids of *B. japonicum* from nodules of soybean roots were isolated using differential centrifugation (the standard bench method) and density gradient centrifugation methods (either sucrose- or Percoll-) under anaerobic conditions in which N_2 fixation was preserved. The relationships between N_2 fixation and respiration, O_2 supply, O_2 demand, substrate (mainly malate) transport and metabolism in bacteroids were investigated using the flow chamber system. In related experiments, the primary products of N_2 fixation which leave the bacteroids were investigated using a ^{15}N-labelling technique in a closed shaken system and other biochemical methods.

In the flow chamber experiments, the rates at which O_2 was supplied to bacteroids in the chamber were varied by (a) changing the flow rate of reaction medium through the chamber; (b) by changing the $[O_{2\ free}]$ in the inflowing reaction medium by using either 3-5% (v/v) or 100% air in the gas mixture above the stirred reaction medium in two reservoir flasks; (c) by successively withdrawing bacteroids from the chamber, thus increasing the supply of O_2 per bacteroid to those remaining in the chamber. The results showed that the rate of O_2 supply regulates respiratory demand for O_2 by bacteroids rather than the O_2 concentration present in the reaction system. Respiration is always coupled to N_2 fixation.

Uptake of malate by bacteroids withdrawn from the flow chamber was measured under microaerobic conditions. Malate uptake by these N_2-fixing bacteroids was lower than that by bacteroids isolated under aerobic conditions, which eliminate N_2 fixation of bacteroids, but is closely correlated with bacteroid respiration rates. When respiration was increased by an increase in O_2 supply, malate uptake by bacteroids was also increased. This suggested that transport of malate through the bacteroid membrane is also regulated by O_2 supply, but indirectly. Higher uptake by bacteroids under aerobic conditions was observed because respiration was enhanced by the high availability of

O_2, but the fast uptake of malate by bacteroids driven by the abnormal respiration rates may not reflect the reality of malate demand *in vivo* by bacteroids when N_2 fixation by bacteroids is fully coupled.

The results of ^{15}N labelling experiments and other biochemical assays once again demonstrated that ammonia is the principal significant ^{15}N labelled product of N_2 fixation accumulated during 30 min in shaken assays with 0.008-0.01 atm O_2. Alanine although sometimes found in low concentrations in the flow chamber reactions, was not labelled with ^{15}N in shaken closed system experiments. No evidence could be obtained from the other biochemical assays, either. Therefore, it is concluded that these and earlier results were not due to contamination with host cytosolic enzymes as suggested by Waters *et al.* (Proc. Natl. Aca. Sci. 95, 1998, pp 12038-12042).

Malate transported into bacteroids is oxidized in a modified TCA cycle present in bacteroids. The results of flow chamber experiments with a *sucA* mutant (lacking α-ketoglutarate dehydrogenase) showed that respiratory demand for O_2 by the mutant bacteroids is regulated by O_2 supply in the same way as the wild-type. Despite differences in other symbiotic properties, rates of nitrogen fixation by the mutant bacteroids, based on the bacteroid dry weight, appeared to be the same as in the wild-type. Also N_2 fixation was closely coupled with respiration in the same manner in both mutant bacteroids and wild type bacteroids. These results and other supporting data, strongly support the conclusion that there is an alternative pathway of the TCA cycle in bacteroids, which enables the missing step in the mutant to be by-passed with sufficient activity to support metabolism of transported malate.

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