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Metabolic Capacity of Purified Vesicles from *Frankia* EAN1pec

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Abstract

The actinorhizal bacteria *Frankia* produce vesicles, specialized cell structures that are the site of nitrogen fixation. Vesicles were isolated and purified from *Frankia* strain EAN1pec grown in culture. The physiological properties of these purified vesicles were characterized with respect to their metabolic capacity and compared to that of the intact mycelium. Our results indicate that the vesicle is unable to generate ATP. When compared on a protein basis, the total adenylate nucleotide pool of intact mycelium was 10-fold higher than total pool obtained with purified vesicles. Purified vesicles exhibit low rates of endogenous respiration, about 5-fold lower than the rates obtained with intact mycelia. The addition of succinate increased vesicle respiration by 1.6-fold, but several other carbon sources had no effect on respiration. Vesicle respiration showed a degree of insensitivity to cyanide. The addition of succinate, a respirable substrate, did not increase the energy charge or ATP level of purified vesicles. In the case of intact mycelium controls, the absence or presence of an energy source directly affected the energy charge and ATP levels. These results combined with those of previous studies indicate that the vesicle is dependent on the ATP supplied from the hyphae to which they are attached.

Keywords: Actinorhizal symbiosis, bioenergetics, nitrogen fixation, ATP

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1. Introduction

Members of the genus *Frankia* are filamentous actinomycetales that infect roots and induce nodule formation in a variety of woody dicotyledonous plants, leading to a symbiotic nitrogen-fixing association (for review see Benson and Silvester, 1993; Wall 2000). These bacteria are developmentally complex forming three cell types: vegetative hyphae, spores located in sporangia and the unique lipid-enveloped cellular structures, termed vesicles (Benson and Silvester, 1993). Vesicles are formed inside of the plant cells of the nodule or in culture under nitrogen limiting conditions and act as specialized structures for the nitrogen fixation process (for review see Benson and Silvester, 1993; Huss-Danell, 1997). Their shape is strain-dependent and host-plant-influenced. Vesicles are formed terminally on short side branches of hyphae that have a septum near their base. The mature vesicle is surrounded by envelope that extends down the stalk of the vesicle past the basal septum which separates the vesicle from the hyphae.

Techniques have been developed for the isolation and purification of intact vesicles from *Frankia* grown in culture (Noridge and Benson, 1986; Tisa and Ensign, 1987c). These purified vesicles retain nitrogenase activity. Initial investigations on the properties of purified vesicles have focused on nitrogen metabolism (Noridge and Benson, 1986; Schultz and Benson, 1989; Tisa and Ensign, 1987c). Carbon metabolism and bioenergetic properties of vesicles have been studied only briefly (Tisa and Ensign, 1987c). The nature of the *in situ* reductant and the source of energy necessary for nitrogen fixation by *Frankia* have not been determined. *Frankia* possess an adenylate nucleotide transport (ADP-ATP translocation) system, which exchanges ATP for ADP (Tisa and Ensign, 1988). This translocase may function to supply the vesicle with ATP for nitrogenase and to remove ADP. This hypothesis would suggest that the vesicle is dependent on the mycelia for the energy to drive nitrogen fixation. The major focus of this study was to measure the catabolic capacity of purified vesicles of *Frankia* EAN1pec and to determine if the vesicle is capable of ATP production. Vesicles were isolated and purified from *Frankia* strain EAN1pec grown in culture. The physiological properties of these purified vesicles were investigated and compared to the intact filamentous form of *Frankia* (hereafter referred to as intact mycelium). Our results described in this communication indicate that the vesicle is unable to generate ATP.

2. Materials and Methods

Organism and growth conditions

Frankia strains Cc1.17 (Meesters et al., 1985), CN3 (Mirza et al, 1994), CpI1

succinate variant [CpI1-S] (Callaham et al., 1978; Tisa et al., 1983), EAN1pec (Lalonde et al., 1981), EuI1c (Baker et al., 1980) and QA3 (Hafeez et al., 1984) were grown and maintained in basal growth medium with NH_4Cl as the nitrogen source, as described previously (Tisa and Ensign, 1987a,c). Strain EAN1pec was the primary strain that was used for the vesicle purification study while the other strains were used in the nucleotide pool analysis experiments on intact mycelium for comparative purposes.

Large scale batch cultures of strain EAN1pec were obtained by growing cells in a carboy with 15 liters of medium containing 20 mM succinate or 20 mM fructose with limited NH_4Cl (0.5 mM), as described previously (Tisa and Ensign, 1987c). Under these conditions, the cells depleted their supply of NH_4Cl after 7 to 8 days of growth and were growing with N_2 as the nitrogen source when harvested.

Vesicle isolation and purification

Vesicles were isolated and purified as described previously (Tisa and Ensign, 1987c) except that anaerobic techniques were not required or used. Freshly harvested or frozen cells were washed 2× with TM buffer (25 mM Tris-HCl/0.5 M mannitol buffer pH 7.4) at 20°C. Vesicles were isolated by passing the washed culture through a French pressure cell at 69 to 138 MPa at 4°C. As a result of this treatment the mycelia are completely disrupted but the vesicles remain intact. Vesicles were purified from the cellular debris by a series of low speed centrifugations at 20°C. The numbers of vesicles were counted by using a Petroff-Hausser counting chamber with a phase-contrast microscope at a magnification of ×400.

Nucleotide pool analysis

The nucleotide pool extracts of purified vesicles and intact mycelia were quantified by high-pressure liquid chromatography analysis. The total nucleotide pools were extracted with 2.5 ml of 0.25 M KOH at 0°C for 10 min. The samples were then centrifuged, and the supernatant fluids were analyzed for nucleotide content by HPLC (Stocchi et al., 1985). Nucleotide peaks were identified by co-migration and they were confirmed by "spiking" samples with an individual nucleotide. Adenylate nucleotide levels were expressed as nmoles/mg protein or nmoles/10^6 vesicles. The energy status of cells was expressed as energy charge [(ATP + 1/2ADP) / (ATP + ADP + AMP)].

Respiratory studies

Oxygen consumption by purified vesicles and intact mycelium was measured

with a Clarke oxygen electrode at 28°C. The 2.0-ml samples of cell or vesicle suspension in growth medium lacking a carbon source were mixed with small (3-mm-long) stirring magnets, and samples were monitored continuously.

Enzyme assays

Crude extracts for enzyme assays were prepared from 1) purified vesicles, 2) the vegetative hyphal fraction of N_2-grown mycelia, and 3) NH_4Cl-grown mycelia. Vesicles were broken by agitation with glass beads as previously described (Tisa and Ensign, 1987c). Intact mycelia were disrupted by passage through a French pressure cell at 69 MPa The disrupted mycelia and vesicles were centrifuged at 20,000 × g for 20 min to remove cellular debris, and the supernatant was used for the enzyme assays.

Isocitrate dehydrogenase and malate dehydrogenase activities were determined spectrophotometrically as described previously (Reeves et al., 1971).

Other analytical methods

Total protein was measured by a modified Lowry procedure (Daniels et al., 1994). Cellular dry weights were determined as described previously (Tisa and Ensign, 1987a).

3. Results

Measurement of nucleotide levels in vesicles

The nucleotide pools of purified vesicles and intact mycelia were extracted and the adenylate pool was analyzed by HPLC (Table 1). When compared on a protein basis, the total adenylate nucleotide pool of intact mycelia was about 10-fold higher than the total pool obtained with purified vesicles. Nitrogen-fixing cultures are reported to have lower energy charge of 0.60–0.65 compared to values of 0.70–0.80 for cultures growing on fixed nitrogen sources (Ludden, 1991). Energy charge values for NH_4Cl-grown intact mycelia from several *Frankia* strains were similar in the range of 0.70–0.80 (Table 1). The energy charge of purified vesicles was about 0.55, while the energy charge of intact mycelium had values above 0.70. These results indicate that the energy-state of purified vesicles is lower than intact mycelium. Similar results are obtained when we compare the % ATP in the total adenylate pool for intact mycelium and purified vesicles.

Determination the respiratory capacity of the vesicle

Purified vesicles exhibited low rates of endogenous respiration (Table 2). These rates were about 5-fold lower than the rates obtained with intact mycelium. The endogenous rate of respiration for NH_4Cl-grown or N_2-grown intact mycelium is about 250 nmoles O_2/mg (dry wt)/h, while the endogenous rate of purified vesicles is about 50 nmoles O_2/mg (dry wt)/h. The addition of succinate resulted in an increase in the vesicle respiratory rate. The specific activity of the respiration rates of purified vesicles was still lower than the rates obtained with intact mycelium. However, this value represents a 1.6-fold increase in respiration over the endogenous rate. With the addition of succinate, intact mycelia exhibit a 1.3- to 1.8-fold increase in their rates of respiration. Several other potential substrates (fructose, malate, propionate, acetate, pyruvate, and mannitol) did not significantly increase the respiration rate of vesicles, but they did increase the rate of intact mycelia (data not shown). *Frankia* strain EAN1pec has inducible and constitutive oxidation systems (Tisa et al., 1983). Succinate respiration is constitutive, while sugars and sugar alcohol respiration are inducible. Purified vesicles from fructose-grown cultures only respired succinate, while intact mycelia respired fructose, succinate, malate, propionate, acetate and mannitol (data not shown). These results indicate that vesicles respiration used succinate but were unable to use several substrates which were respired by the hyphae. N_2-grown cells possess a cyanide-insensitive respiratory system that is absent in NH_4Cl-grown *Frankia* (Tisa and Ensign, 1987b). Cyanide inhibited vesicle endogenous respiration by about 80% (Table 2). These results would suggest a portion of the vesicle respiration occurs via a cyanide-insensitive respiratory system.

Enzyme content of intact vesicles

Enzyme activities were measured for two TCA enzymes (Table 3). Vesicle enzyme activity levels were similar to those obtained with intact mycelia and vegetative hyphae. Enzyme activities were only detected when purified vesicles were disrupted. This would support the idea that the vesicles are intact undamaged units. These results also suggest that the vesicle is capable of catabolic activities. This catabolic activity may be involved in the generation of reductant and ATP that are necessary for nitrogen fixation.

Determination of ATP production

In our controls, the energy charge and ATP levels of intact mycelium were directly affected by the presence of an energy source (Table 4). Removal of an energy source lowered cellular ATP levels and the energy charge of the cells.

Table 1. Analysis of the adenylate nucleotide pools of *Frankia* strains. Nucleotides levels are expressed as nmoles/mg protein. Values represent the average of 3–7 measurements ± standard deviation.

	ATP	ADP	AMP	% ATP in total adenylate pool	Energy charge[a]
NH_4Cl-grown intact mycelium:					
Strain CN3	4.72 ± 0.33	1.58 ± 0.28	1.58 ± 0.12	59.6 ± 0.4	0.70 ± 0.02
Strain CcI.17	4.33 ± 1.74	0.66 ± 0.33	0.28 ± 0.14	82.7 ± 2.5	0.89 ± 0.02
Strain CpI1-S	1.00 ± 0.7	0.71 ± 0.60	0.29 ± 0.08	48.8 ± 18.9	0.69 ± 0.07
Strain EuI1c	23.78 ± 6.58	3.54 ± 1.08	8.70 ± 1.18	58.2 ± 16.7	0.71 ± 0.12
Strain QA3	3.05 ± 0.21	0.66 ± 0.10	0.57 ± 0.14	71.2 ± 2.2	0.80 ± 0.03
Strain EAN1pec	18.49 ± 4.70	3.56 ± 1.09	7.94 ± 1.07	61 ± 5	0.68 ± 0.05
N_2-grown intact mycelium:					
Strain EAN1pec	18.26 ± 6.39	8.33 ± 1.45	4.93 ± 1.21	58 ± 6	0.71 ± 0.06
Purified vesicles:					
Strain EAN1pec	1.24 ± 0.18	1.21 ± 0.25	0.72 ± 0.15	39 ± 4	0.55 ± 0.08

[a]Energy charge = [(ATP + 1/2ADP) / (ATP + ADP + AMP)].

Table 2. Oxygen consumption by intact mycelium and purified vesicles of *Frankia* EAN1pec.

	Respiration rate[a] (nmoles O_2/mg (dry wt)/h)			
	Intact mycelium[b]		Purified vesicles	
Condition	NH_4Cl-grown	N_2-grown	Succinate-grown	Fructose-grown
Endogenous rate (no substrate)	228 ± 25	226 ± 60	53.4 ± 6.5	50.8 ± 6.8
+ 20 mM succinate	442 ± 110	348 ± 90	81.2 ± 7.4	82.3 ± 6.1
+ 20 mM succinate and 2 mM KCN	0	97 ± 6	10.0 ± 4.2	7.4 ± 3.1

[a]Values represent the average of 3–7 experiments ± standard deviation. [b]Cultures grown in succinate medium.

Table 3. TCA enzyme activities in vesicles and intact mycelium of *Frankia* strain EAN1pec. Enzyme activity was expressed as units/min/mg protein. Values represent the average of 3 measurements ± standard deviation.

Crude extracts	Isocitrate DH activity	Malate DH activity
Vegetative hyphal extracts	800 ± 14	820 ± 22
Untreated purified vesicles	0	0
Glass bead disrupted vesicles	790 ± 35	840 ± 67

Table 4. Effect of different environmental conditions on the energy status of intact mycelium and purified vesicles. Values represent the average of 2–4 measurements ± standard deviation. All cultures were grown in succinate growth medium. In experiment #2, intact mycelium was washed three times with MOPS-phosphate buffer and finally suspended in the same buffer with and without succinate. In experiment #3, intact mycelium was treated with the same procedures that were used to purify vesicles except they were not passed through a French pressure cell.

Intact mycelium	% ATP in total adenylate pool	Energy charge[a]
Experiment #1		
Control (untreated cells)	53 ± 5	0.71 ± 0.02
De-energized cells[b]	15 ± 9	0.29 ± 0.01
Experiment #2		
Before treatment	49 ± 1	0.65 ± 0.07
Control (no substrate)	46 ± 6	0.67 ± 0.06
+ 20 mM succinate	55 ± 2	0.84 ± 0.18
Experiment #3		
Before treatment	56 ± 5	0.67 ± 0.07
Control (no substrate)	29 ± 4	0.51 ± 0.02
+ 20 mM succinate	63 ± 7	0.74 ± 0.09
Purified vesicles incubated 30 min		
Control (no substrate)	39 ± 4	0.58 ± 0.08
+ 20 mM succinate	38 ± 3	0.53 ± 0.06
+ 2 mM KCN	38 ± 4	0.54 ± 0.06
+ 20 mM succinate and 2 mM KCN	39 ± 3	0.53 ± 0.07
+ 200 µM CCCP[c]	37 ± 4	0.56 ± 0.08

[a]Energy charge = [(ATP + 1/2ADP) / (ATP + ADP + AMP)]. [b]Intact mycelium was incubated overnight at 4°C in growth medium lacking a carbon source. [c]CCCP, carbonyl cyanide-p-trifluoromethylhydrazone.

The addition of an energy source to starved or de-energized intact mycelium resulted in an increase in the ATP pool and energy charge. However, the addition of an energy source did not affect the energy charge and ATP levels of purified vesicles (Table 4), they remained constant. These results indicate that vesicles from *Frankia* EAN1pec are incapable of ATP production.

4. Discussion

Purified vesicles are intact vesicles

We have several lines of evidence which indicate that purified vesicles are intact structures that are not damaged. First, treating purified vesicles with detergents and other permeabilizing agents results in the loss of endogenous and succinate-stimulated respiration (data not shown). These types of treatments increased the permeability of purified vesicles to dithionite and these treated-purified vesicles show increased nitrogenase activity (Tisa and Ensign, 1987c). Second, TCA enzymes activities detected in purified vesicles were measurable only in disrupted samples (Table 4). Intact purified vesicles gave no detectable measurements using these assays. Third, nitrogenase activity is associated with the vesicle and the enzyme as detected by enzyme activity does not leak from purified vesicles (Tisa and Ensign, 1987c). Vesicles that are isolated and purified by the use of French pressure cell disruption and differential centrifugation do not release nitrogenase from the vesicle during the purification process (Tisa and Ensign, 1987c). Glass bead disruption of purified vesicles releases nitrogenase (Tisa and Ensign, 1987c). Digestion of the vesicles with lysozyme also releases nitrogenase. Lysozyme digestion appears to affect the base of the stalk of the vesicle. The above lines of evidence indicate that purified vesicles are intact undamaged structures and they should have the potential to generate ATP. However, vesicles are not freely permeable. Substrates do not free diffuse into the vesicle, but are probably transported inside the structure at the base of the stem. The addition of succinate increased respiration by purified vesicles suggesting that vesicles are capable of transporting succinate inside the structure.

Vesicles are incapable of ATP production

The key question that we wanted to answer was whether or not the vesicles are capable of generating their own ATP for nitrogen fixation. To answer the question, the effect of respirable substrates on the ATP levels and energy charge of vesicles and intact mycelium was determined. Several different experimental conditions were tested on vesicles and intact mycelium (our

designated control). After these treatments, the nucleotide pools of intact mycelium and purified vesicles were extracted and analyzed by HPLC. Since the total adenylate pool of intact mycelium and vesicles differed, we looked for changes in both the % of ATP in the total adenylate pool and the energy charge. Although purified vesicles are intact structures, our results indicate that the vesicle is incapable of ATP production. Vesicles respired succinate (Table 2) but this respiration did not result in an increase in ATP production or a change in energy charge (Table 5). Control experiments with intact mycelium show that ATP levels and energy charge increased in the presence of succinate (Table 4) indicating that the ATP increase should be detectable. Since the vesicle appears to be unable to generate its own ATP for nitrogen fixation, this would indicate that vesicles are dependent on the mycelia for energy for nitrogen fixation.

Our previous work has shown that the adenylate nucleotide pool of purified vesicles is changed after 1 h incubation with 1.0 mM ATP (Tisa and Ensign, 1988). The resulting adenylate nucleotide pool of the treated vesicles contained only ATP and no detectable ADP or AMP. This shows that translocase activity is able to change the energy-state of the vesicle.

Relationship to in planta studies

Another approach toward studying *Frankia* physiologically involves the isolation of vesicle clusters from root nodules that are free of plant materials (for review see Huss-Danell, 1997). Several enzymes for sugar degradation have been detected in these preparations (Akkermans et al,, 1981; Huss-Danell et al., 1982; Lopez and Torrey, 1985; Vikman and Huss-Danell, 1987). The complex origin of these vesicle preparations brings into question their purity and makes it difficult to interpret the results of such studies. In our study, TCA enzyme activities for purified vesicles (Table 4) were similar to the values reported for symbiotic vesicles. Respiratory studies with vesicle cluster preparations are also complicated by presence of hyphae and some plant materials (Akkermans et al., 1981; Huss-Danell et al., 1982; Lopez and Torrey, 1985; Vikman and Huss-Danell, 1987ab, 1991, 1992). Improved techniques have yielded isolated symbiotic vesicles free of hyphae (Vikman and Huss-Danell, 1992). These isolated vesicles respire 6-phosphogluconate, NADH, and malate + glutamate. The rate of respiration for intact vesicles from *in culture* (Table 2) was similar to the rate of respiration for symbiotic vesicles.

A malate-aspartate shuttle has been proposed as a means of transporting reducing equivalents into the vesicle from the plant cytoplasm (Akkermans et al., 1981). Since malate dehydrogenase and glutamate-oxaloacetate aminotransferase activities are detected in vesicle clusters (Akkermans et al.,

1981; Huss-Danell, 1982; Vikman and Huss-Danell, 1987, 1991; Vikman, 1992), one proposed explanation for the observed respiration of malate, glutamate and NAD^+ by symbiotic vesicles is the presence of the malate-aspartate shuttle. It has been suggested that the permeability of symbiotic vesicles to low-molecular weight molecules including NAD^+, phosphorylated hexoses and ATP may reflect intrinsic membrane instability or difference between symbiotic vesicle and hyphal cell types (Benson and Eveliegh, 1979). We have also detected malate dehydrogenase activity in purified vesicles (Table 3). However, vesicle enzyme activity was only detected after the integrity of the intact vesicle was disrupted. These vesicles are intact sealed structures and do not appear to be damaged (see discussion above). One possible explanation for the above observations is that symbiotic vesicles are more permeability than vesicles developed in culture. The plant cell environment may be more conducive to maintain a higher permeability, which could be lethal in culture. This hypothesis requires further testing including the determination how physiologically similar purified symbiotic vesicles are to purified vesicles formed in culture?

Vesicle respiration

Frankia grown under N_2 conditions contains two respiratory systems: one CN-sensitive and another CN-insensitive (Tisa and Ensign, 1987b). NH_4-repressed cells do not exhibit cyanide-insensitive respiration. In this study, respiratory experiments indicate that the vesicle possesses a CN-insensitive respiratory system (Table 2). One hypothesis is that cyanide-insensitive respiration is involved in the protection of nitrogenase from oxygen. The thick envelope of the vesicle acts as a diffusion barrier to protect the nitrogenase from oxygen inactivation. Low levels of oxygen would be consumed by the vesicle respiratory system to provide an anaerobic environment. Analysis of vesicles clusters isolated from root nodules of *Alnus rubra* detected the presence of cytochrome a–a_3, c, and o (Ching et al., 1983). These cytochromes were suggested to be involved in the mechanism of oxygen protection for nitrogenase. This hypothesis also requires further testing.

What does the absence of ATP production mean?

Catabolic capacity of the vesicle was investigated in this study. Purified vesicles respired succinate and exhibited malate dehydrogenase and isocitrate dehydrogenase activities that were comparable to those vegetative hyphae. Both the enzyme activities and respiration rates were similar to the values reported with vesicle clusters. These catabolic activities could be involved in

the generation of the low potential reductant that is required for nitrogen fixation. The lack of ATP production by purified vesicles implies that the vesicles are obtaining their ATP from another source. This idea would suggest that the ATP/ADP translocase plays a key role in the vesicle.

What is the potential role of the ATP/ADP translocase in vesicle function?

Our current working model is that the role of the translocase is to supply the ATP requirement for nitrogen fixation. The transport system might be involved in communication between mycelia and vesicles of *Frankia*. It is clear that there is a membrane barrier separating mycelial and vesicle cytoplasm (Lancelle et al., 1985). We hypothesize that the translocase functions to supply energy directly in the form of ATP to nitrogenase inside the vesicle. The system would remove ADP from the vesicles so that ADP would not accumulate to inhibitory levels. High levels of ADP in vitro (Lowe et al., 1980) inhibit the activity of purified nitrogenase. The presence of the translocase in *Frankia* is significant and may also be involved in transporting energy from mitochondria in plant tissue into *Frankia* vesicles in root nodules. The vesicles are compartmentalized by internal septation or ingrowth of the cell membrane (Huss-Danell and Bergman, 1990; Newcomb and Wood, 1987). It is possible that nitrogenase is also compartmentalized. The translocase would function to supply ATP to the compartmentalized nitrogenase. The data from our current study suggest that vesicles may be dependent on the mycelia for energy for nitrogen fixation. We are presently investigating the roles of ATP translocase and cyanide-insensitive respiratory system in the vesicle.

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