7-1-1988

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Comments

This is an article published by American Society for Microbiology in Journal of Bacteriology in 1988, available online:

https://dx.doi.org/10.1128/jb.170.7.3053-3057.1988

Recommended Citation

Tisa, L.S. and J.C. Ensign. 1988. Evidence for adenylate nucleotide transport (ATP-ADP translocation) in vesicles of Frankia sp. strain EAN1pec. J. Bacteriol. 170:3053-3057.

JOURNAL OF BACTERIOLOGY, July 1988, p. 3053–3057
0021-9193/88/073053-05$02.00/0

Vol. 170, No. 7

Evidence for Adenylate Nucleotide Transport (ATP-ADP Translocation) in Vesicles of *Frankia* sp. Strain $EAN1_{pec}$

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Received 29 January 1988/Accepted 23 March 1988

Atractyloside and carboxyatractyloside partially inhibited nitrogenase activity (acetylene reduction) by isolated vesicles of *Frankia* strain $EAN1_{pec}$. Extracts of disrupted vesicles showed nitrogenase activity that was not affected by the inhibitors. The vesicles accumulated ATP by an atractyloside-sensitive mechanism. This inhibition of ATP uptake was reversed when vesicles were permeabilized by detergent. Uptake of ATP was inhibited by excess ATP and ADP, but not AMP or adenosine, and by a calcium-dependent ATPase inhibitor. Uptake was stimulated by calcium ions. Accumulation of ATP was accompanied by release of ADP and AMP from the vesicles. The ATP taken up by vesicles and cells grown with N_2 as the nitrogen source was found in the corresponding cell pools only as ATP. The data indicate activity of an ATP-ADP translocase system in vesicles of this organism. The role of ATP translocation in the symbiosis between *Frankia* strain $EAN1_{pec}$ and plant root nodules is discussed.

Nitrogen-fixing actinomycetes of the genus *Frankia* induce the formation of root nodules in a variety of woody dicotyledonous plants. The bacteria form clusters of spherical structures termed vesicles inside the plant cells of the nodule (19, 26, 31). The appearance of nitrogenase activity in laboratory cultures of *Frankia* spp. follows formation of the vesicles (17, 23, 28, 32). Studies using isolated vesicles (23, 29) and immunocytological methods (16–18) indicate that vesicles are the site of nitrogenase activity.

Cell extract preparations of alder nodules require ATP and reducing equivalents for nitrogenase activity (3). The sources of energy and reductant for nitrogen fixation by *Frankia* spp. in the nodules are not known. Akkermans et al. (1, 2) showed that enzymes associated with the malate-aspartate shuttle system were present in vesicle cluster preparations from nodules and proposed that this system provides the reducing equivalents for nitrogenase. We previously reported (29) that isolated vesicles are capable of nitrogen fixation when they are incubated in the presence of dithionite and Mg-ATP. This observation suggests that vesicles may take up intact ATP to supply energy for nitrogenase activity. This is an interesting idea in light of reports that plant mitochondria are in close association with vesicle clusters within the root nodules (1, 2, 14).

The excretion of ATP coupled with ADP uptake is catalyzed by an adenylate nucleotide transport system (termed ATP-ADP translocase) located in the inner membranes of mitochondria and chloroplasts (10, 34, 38). Translocase activity is involved in ATP uptake by the intracellular parasites *Rickettsia* (36, 37) and *Chlamydia* (9) cells. Intracellular membranes of *Methanobacterium thermoautotrophicum* were reported to contain ATP-ADP translocase activity and were postulated to function as "methanochondria" in providing ATP to the cell (5, 6). Krämer and Schönheit (12) recently reported that these intracellular membranes do not exchange (translocate) ATP-ADP but bind ADP and ATP to specific sites at the inner side of the cell membrane. Transport of ATP by *Bdellovibrio* spp. occurs by an energy-dependent process not involving an ATP-ADP exchange mechanism (25).

In this paper, we report evidence for an ATP-ADP translocase system in isolated vesicles of *Frankia* strain $EAN1_{pec}$ (*Frankia* strain EAN).

MATERIALS AND METHODS

Organism and culture conditions. *Frankia* strain EAN (*Frankia* registry number ULQ13100144 [13]) was obtained from M. Lalonde, Laval University, Quebec, Canada. Cultures were grown and maintained in basal growth medium under nitrogen-repressed conditions with NH_4Cl as the nitrogen source, as described previously (28). Large-scale batch cultures of cells derepressed for nitrogenase were obtained by growing cells in a carboy with 15 liters of medium as described previously (29). Under these conditions, the cells depleted their supply of NH_4 after 7 to 8 days of growth and were growing with N_2 as the nitrogen source when they were harvested.

Vesicle isolation and purification. Cells were incubated under nitrogen-depressed conditions with N_2 as the nitrogen source to induce vesicle development and nitrogenase activity. Cells grown for 14 days in medium containing NH_4Cl as the nitrogen source were harvested and washed three times with MP buffer (20 mM morpholinepropanesulfonic acid [MOPS] and 10 mM KH_2PO_4 buffer at [pH 6.8]). The cells were then inoculated into succinate growth medium with N_2 as the sole nitrogen source and incubated for 4 days at 25°C before harvest.

Vesicles were isolated from the N_2-grown cells and purified by the procedure of Tisa and Ensign (29). Cells were passed through a French pressure cell at 10,000 to 12,000 lb/in^2 at 4°C to disrupt mycelia and separate vesicles. The vesicles were purified from cellular debris by a series of low-speed centrifugations at 20°C. For some experiments, stringent anaerobic techniques were used to ensure that there was no exposure to oxygen.

Nitrogenase activity. Nitrogenase activity was measured by the acetylene reduction assay as described previously (29). Whole-cell activity was assayed at 25°C at atmospheric partial pressures of oxygen. Unless otherwise noted, isolated vesicles were assayed for nitrogenase activity anaero-

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bically and were supplied Mg^{2+} ions, an ATP-regenerating system and sodium dithionite were supplied as sources of energy and reductant, respectively.

Accumulation of radiolabeled ATP. The accumulation of [U-^{14}C]ATP or [α-^{32}P]ATP by isolated vesicles was assayed by suspending vesicles in 1.0 ml of 25 mM Tris hydrochloride buffer (pH 7.4). For some experiments, 1.0 mM $CaCl_2$ was added. The reaction was started by the addition of labeled ATP, and the mixture was incubated aerobically at 25°C. At zero time and after different time intervals, 200-μl samples were removed, filtered through 0.2-μm-pore-size membrane filters (Millipore Corp.), and washed with 20 ml of 1.0 nM unlabeled ATP. The elapsed time for sampling and washing was 1.5 to 2 min. The filters were transferred to vials containing 10 ml of Aquasol, and their radioactive contents were determined with a scintillation counter. The rates of accumulation of label were determined during the initial 5-min period, when the rates were linear. Rates are expressed as picomoles of ATP accumulated per minute per milligram of protein. Zero-time values, which were less than 100 pmol/mg of protein, were subtracted from subsequent determinations. To distinguish between uptake and nonspecific binding of ATP, determinations were made as described above during incubation at 0°C and by using vesicles that had been heated at 90°C for 15 min. In neither case was the uptake of label greater than that in the zero-time sample.

The accumulation of labeled ATP by whole cells was also assayed as described above, except that the cells were incubated in growth medium lacking a carbon source.

Competition experiments testing the effects of ATP, ADP, AMP, adenosine, and adenine on the accumulation of labeled ATP were performed as described above. These compounds were added at the start of the reaction. Inhibitors, when used, were preincubated with the vesicles and whole cells for 5 min before the start of the reaction.

To measure ATP efflux, whole cells or isolated vesicles were allowed to accumulate radiolabeled ATP by incubating the samples for 30 min as described above. The samples were collected by centrifugation and washed with MP buffer. These samples were suspended in 1.0-ml portions of MP buffer containing one test substance each. At different time intervals, 200-μl samples were removed and filtered through 0.2-μm-pore-size membrane filters. The filtrates were collected, and the amount of radioactive label was measured. The filters were washed with MP buffer, dried, and counted to determine the amount of radioactivity retained by the cells or vesicles.

Nucleotide pool analyses. To determine the fate of accumulated ATP, cells or isolated vesicles were incubated with 10 μCi of [α-^{32}P] ATP per ml for 30 min at 25°C as described above. The samples were collected and washed with MP buffer to remove nonspecifically bound label. The adenylate pool of the cells was extracted with 0.5 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 thin-layer chromatography by the method of Mangold (15). Samples (20 μl) were applied to cellulose MN 300 plates (Macherey-Nagel and Co.) and separated in a solvent system of *n*-butanol–acetone–acetic acid–5% aqueous ammonium–H_2O (9:3:2:2:4). Authentic ATP and ADP standards were also applied to the plates. After development for 2 h or until the solvent front reached a level 2 in. (ca. 5 cm) below the top of the plate, the regions corresponding to ATP and ADP were scraped from the plate and radioactivity was determined by using Aquasol as a scintillation cocktail.

The nucleotide pool extracts of purified vesicles were quantified by high-pressure liquid chromatography analysis. Vesicles incubated with 2.0 mM $MgCl_2$–1.0 mM ATP–20.0 mM dithionite at 30°C for 1.0 h were centrifuged and washed once with MP buffer. Their nucleotide pools were extracted with cold 0.25 M KOH and analyzed by high-pressure liquid chromatography (27). The supernatant fluid which remained after removal of vesicles by filtration was also analyzed by high-pressure liquid chromatography.

TABLE 1. Effects of atractyloside and carboxyatractyloside on acetylene reduction by isolated vesicles

Inhibitor (mM)	Nitrogenase activity[a]	
	nmol/h per mg of protein	% Inhibition
None	615	
Atractyloside (2.5)	187	70
Atractyloside (1.0)	343	44
Atractyloside (1.0), preincubated[b]	184	70
Carboxyatractyloside (2.5)	258	58
Carboxyatractyloside (1.0)	308	50
Atractyloside (1.0) + ATP (5.0)	539	12

[a] Acetylene reduction was assayed at 25°C with 2.5 mM ATP, 5.0 mM $MgCl_2$, and 100 mM dithionite under an argon atmosphere. Vesicles were isolated and purified as described in Materials and Methods. The inhibitors were added anaerobically at the start of the assay.

[b] Isolated vesicles were preincubated with the inhibitor for 15 min before the start of the reaction.

Total protein determination. Total protein was measured by the Bradford procedure (4) after samples were solubilized by heating for 15 min at 90°C in 1.0 N NaOH.

RESULTS

Effects of atractyloside and carboxyatractyloside on acetylene reduction by isolated vesicles. Atractyloside and carboxyatractyloside are specific inhibitors of mitochondrial ATP translocation (34, 35). The effects of these inhibitors on nitrogenase activity of isolated vesicles were tested by adding them to the vesicles incubated with ATP and dithionite (Table 1). Both inhibitors significantly reduced the rate of acetylene reduction by isolated vesicles but did not totally block activity. Preincubation of vesicles with 1.0 mM atractyloside for 15 min resulted in a 70% inhibition of nitrogenase activity. Inhibition by 1.0 mM atractyloside was reduced but not totally eliminated when the level of ATP was increased to 5.0 mM. The rate of acetylene reduction by vesicles was the same when they were incubated with 2.5 and 5.0 mM ATP (data not shown).

The effect of these inhibitors on cell-free nitrogenase activity was tested. A 20,000 × *g* supernatant fraction of disrupted vesicles contained nitrogenase activity when it was incubated with Mg-ATP and dithionite. The addition of 2.5 mM atractyloside or carboxyatractyloside did not affect the activity (data not shown). Inhibition of nitrogenase activity of intact vesicles but not of vesicle extracts indicates that the inhibitors affect a membrane transport process. The known activity of the inhibitors as ATP transport blockers suggests that they inhibit nitrogenase activity of vesicles by blocking an ATP translocase system.

ATP accumulation studies. A direct test of ATP translocase activity in vesicles was made by determining their ability to accumulate ATP. The vesicles accumulated [^{14}C]ATP at the same rate whether they were incubated aerobically or anaerobically (Table 2). Uptake of ATP was

TABLE 2. [^{14}C]ATP accumulation by isolated vesicles of *Frankia* strain EAN[a]

Condition	ATP accumulation[b]
Anaerobiosis	93.0
Aerobiosis	93.1
Preincubation with 1.0 mM atractyloside[c]	0
Preincubation with 1.0 mM atractyloside + 0.2% CTAB[d]	114.4
Heated vesicles (90°C for 15 min)	0
Incubation at 0°C	1.5

[a] Isolated vesicles were incubated with 23.6 nmol of [α-^{14}C]ATP at 25°C. ATP accumulation was measured by collecting vesicles on filters at different time intervals as described in Materials and Methods.

[b] In picomoles of ATP per minute per milligram of protein.

[c] Isolated vesicles were preincubated with 1.0 mM atractyloside for 30 min at 25°C before the start of the reaction.

[d] Cetyltrimethylammonium bromide (CTAB) was added to atractyloside-treated vesicles to a final concentration of 0.2% before the start of the reaction.

completely inhibited by atractyloside and heat treatment and was insignificant at 0°C. Addition of the detergent CTAB (cetyltrimethylammonium bromide) to the atractyloside-inhibited vesicles resulted in a complete reversal of inhibition. The complete inhibition of ATP uptake by atractyloside observed in this experiment and the only partial inhibition of nitrogenase shown previously (Table 1) are explained by the lower amount of ATP (23.6 nmol) used in the uptake study (2.5 mmol was used in nitrogenase assays).

The kinetics of [^{14}C]ATP uptake is illustrated in Fig. 1. A maximum level of approximately 1.0 nmol of ATP per mg of protein was reached at 5 min and remained nearly constant for the following 25 min. At 20 min (Fig. 1, arrow) samples of vesicles were removed, washed by centrifugation to remove residual external labeled ATP, and then incubated in water or with 250 mM unlabeled adenosine, AMP, ADP, or ATP. The amount of label remaining in the vesicles was determined 5 and 10 min later. The vesicles lost none of their labeled ATP after incubation in water, adenosine, AMP, or

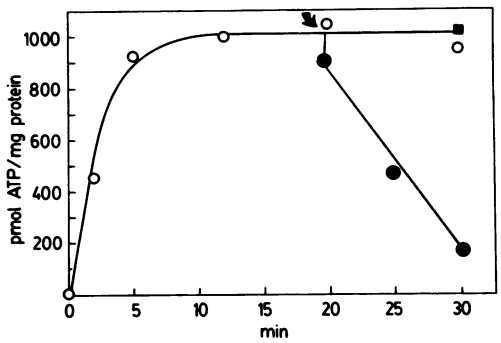

FIG. 1. Accumulation of [U-^{14}C]ATP by vesicles. Vesicles were incubated with 23.5 nmol of [U-^{14}C]ATP and 1.0 mM $CaCl_2$ in 25 mM Tris buffer. Samples removed at various times between 0 and 20 min were collected on membrane filters and washed with 1 mM ATP. At 20 min (arrow), vesicles were washed by centrifugation and suspended in water. Samples were then incubated for an additional 10 min at 25°C in water or with 250 mM unlabeled ATP, AMP, ADP, or adenosine. The amount of label was determined 5 and 10 min later by collecting samples or filters and washing as described above. Symbols: ○, incubation with [U-^{14}C]ATP; ■, incubation with [U-^{14}C]ATP, washing, and incubation for 10 min in water or with unlabeled ADP, AMP, or adenosine; ●, incubation with [U-^{14}C] ATP, washing, and incubation with unlabeled ATP.

TABLE 3. Effects of various compounds on ATP accumulation by isolated vesicles of *Frankia* strain EAN

Compound added	% ATP accumulated[a]
None (control)	100
ATP (125 nmol)	23
ADP (125 nmol)	38
AMP (125 nmol)	107
Adenosine (125 nmol)	102
$MgCl_2$ (250 nmol)	101
$CaCl_2$ (250 nmol)	328
GI (5 μl)	65

[a] Vesicles were incubated at 25°C with 56.5 nmol of [α-^{32}P]ATP. The amount of ATP accumulated was determined as described in Materials and Methods. The control cells accumulated ATP at a rate of 332 pmol/mg of protein per 5 min. For comparative purposes, values are expressed as percentages, with the value for the control (no additions) defined as 100%.

ADP. There was, however, a rapid release of labeled ATP from vesicles incubated with unlabeled ATP. The rate and amount of [α-^{32}P]ATP uptake by vesicles were virtually identical to those of [^{14}C]ATP uptake (data not shown). This indicates that ATP per se is accumulated by the vesicles, which was verified by analyses of the nucleotide pools of vesicles. Approximately 95% of the [α-^{32}P]ATP was recovered as ATP. A small amount of the label (less than 5%) was recovered as ADP.

The total adenylate nucleotide pool of the vesicles contained (in nanomoles per milligram of protein) 0.35 ATP, 0.30 ADP, and 0.31 AMP. Nucleotide pool analyses of vesicles after incubation with 1.0 mM ATP for 1 h revealed the level of ATP to be approximately 1.0 nmol/mg of protein, and no ADP or AMP was detected by the sensitive high-pressure liquid chromatography procedure used. Analyses of the supernatant fluid after removal of vesicles at 1 h revealed the presence of (in nanomoles milliliter) 900 ATP, 28.0 ADP, and 29.2 AMP. It is apparent that ATP taken up by the vesicles was both accumulated and hydrolyzed to ADP and AMP and that these molecules were excreted from the vesicles. The saturation level of ATP in vesicles of 1.0 nmol/mg of protein, which is shown in Fig. 1 to remain constant, agrees with the amount determined by nucleotide pool analyses of vesicles incubated with ATP.

Some compounds that might affect ATP transport were tested for their effect on radiolabeled-ATP accumulation by vesicles. A 2:1 molar excess of unlabeled ATP or ADP reduced labeled-ATP uptake (Table 3). Adenosine, AMP, and magnesium ions did not affect uptake. Uptake of ATP was stimulated threefold by calcium ions and was partially inhibited by GI, an inhibitor of Ca-dependent ATPase activity (8). We previously reported that nitrogenase activity of *Frankia* strain EAN was stimulated by calcium ions and inhibited by GI (29).

The abilities of N_2- and NH_4-grown cells to accumulate ATP were tested. The N_2-grown cells, which are derepressed for nitrogenase and possess large numbers of vesicles, accumulated radiolabeled ATP (Table 4). The ability to accumulate ATP was inhibited by atractyloside and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid] and was stimulated by calcium ions. Removing EGTA by washing did not restore the ability of the N_2-grown cells to accumulate ATP. Adding 1.0 mM $CaCl_2$ to the washed cells resulted in a surprisingly large amount of ATP uptake, amounting to approximately 10 times that resulting from adding calcium to cells not treated with EGTA. We have no explanation for this result, but it is possible that

TABLE 4. [^{14}C]ATP accumulation by whole cells of *Frankia* strain EAN

Condition(s)	ATP accumulation[a] by cells grown with:	
	NH_4Cl	N_2
No additions	0	3.26
+ 500 μM atractyloside	0	0.52
+ 1.0 mM $MgCl_2$	0	3.20
+ 1.0 mM $CaCl_2$	0	5.83
50 mM EGTA[b]	ND[c]	0
50 mM EGTA; MP buffer[d]	ND	0
50 mM EGTA; MP buffer; + 1.0 mM $CaCl_2$ added	ND	60.51

[a] Cells were incubated in growth medium with N_2 or NH_4Cl as the nitrogen source (without a trace salts mixture) with 23.6 nmol of [α-^{14}C]ATP at 25°C. ATP accumulation (in picomoles per minute per milligram of protein) was measured by rapidly collecting samples on filters. The filters were washed and dried, and the content of radioactive label was measured.

[b] Cells were suspended in 50 mM EGTA (pH 7.0) for 5 min.

[c] ND, Not determined.

[d] Cells were suspended in 50 mM EGTA (pH 7.0) for 5 min and then washed three times with MP buffer by centrifugation. The washed cells were incubated as described above before the start of the reaction.

EGTA removes some metal ion that inhibits or retards ATP uptake by the cells.

The ATP taken up by the intact N_2-grown cells was not altered. The rates of uptake of [α-^{32}P]ATP and [U-^{14}C]ATP were the same, and nucleotide pool analyses showed that more than 95% of the label was recovered as ATP.

DISCUSSION

Several kinds of evidence have established that vesicles of *Frankia* strain EAN accumulate ATP by a translocation system. The isolated vesicles rapidly accumulated labeled ATP by a reaction that was blocked by addition of unlabeled ATP and ADP but not by a 1,000-fold excess of adenine, adenosine, or AMP. The accumulated ATP was not modified. Uptake of [α-^{32}P]ATP and [U-^{14}C]ATP occurred at similar rates, and analyses of the nucleotide pools of the [^{32}P]ATP-loaded vesicles showed ATP to be the only labeled component. Vesicles that had accumulated ATP excreted ADP and AMP. The translocase may thereby serve two functions, supply of ATP for nitrogenase and removal of ADP, a potential inhibitor of the enzyme. The observation that the labeled ATP accumulated by vesicles was released only after addition of unlabeled ATP and not after addition of other adenylates is consistent with translocase activity. Atractyloside and carboxyatractyloside, specific inhibitors of mitochondrial ATP-ADP translocation (34, 35), inhibited uptake of ATP by the isolated vesicles. Inhibition by atractyloside involved a membrane transport system, because no inhibition occurred with detergent-treated vesicles. Also, N_2-grown cells, which are capable of nitrogen fixation and which possess functional vesicles, accumulated ATP, whereas NH_4-grown cells did not.

Calcium plays an essential role in nitrogen fixation and vesicle development in *Frankia* strain EAN (30). Calcium is also essential for transport of ATP by isolated vesicles and N_2-grown intact cells (Tables 3 and 4). The most logical explanation for these observations is a calcium transport-linked ATP translocase in the vesicles. Calcium ions are involved in the ATP-ADP translocase system (ATP out, ADP in) of mitochondria (11).

We established in a previous study that the procedures used for removal of vesicles from mycelia and subsequent purification yielded vesicles that are functionally intact (29). *Frankia* vesicles arise from short stalks attached to vegetative mycelia (7, 31, 32). The vesicle wall is a multilaminate structure and may play a direct role as a physical barrier in protecting nitrogenase from oxygen (20, 21, 24, 33). It is unlikely that ATP permeates this thick wall structure. We favor the idea that the ATP-translocating system is associated with the stalk structure and functions in transporting ATP from the metabolizing and energy-generating mycelia into the vesicle. Isolated vesicles are incapable of nitrogen fixation when they are supplied with oxidizable substrates, and they must be supplied with both ATP and reducing power (23, 29). A membrane septum located in the stalk separates the vesicle from the mycelium (22). It is possible that this septum is the site of the translocase. The question of whether the enzyme is specifically localized at this site or present throughout the membrane of the vesicle awaits development of methods for rupturing membranes and isolating them from vesicles.

The existence of an uptake ATP translocation system in *Frankia* spp. may be of special physiological significance with respect to their symbiotic association with plants. The organisms are found as massive clumps of vesicles inside plant cells in close association with mitochondria (1, 2). It is possible that the translocase system of the vesicles takes up the ATP needed to drive nitrogenase directly from the mitochondria. If so, the basis for the *Frankia*-plant symbiosis is quite different from the *Rhizobium*-plant symbiosis.

It is interesting that the ATP-ADP translocase system has been demonstrated in the intracellular parasites *Chlamydia* and *Rickettsia* spp. (9, 36, 37) and in the energy-generating mitochondria and chloroplasts of eucaryotic cells (10, 34, 38). *Bdellovibrio* spp., the intraperiplasmic parasites of gram-negative bacteria, accumulate ATP by a mechanism that is not an ATP translocase (25). The rate of ATP uptake (220 pmol of ATP/min per mg of protein) by isolated vesicles of *Frankia* spp. is similar to the rates reported for cells of *Rickettsia prowazekii*, *Clamydia psittaci*, and *Bdellovibrio bacteriovorus* (9, 25, 37).

ACKNOWLEDGMENTS

This investigation was supported by U.S. Department of Agriculture grant 84CRCR1-1408, by Public Health Service grant AI-13948 from the National Institutes of Health, and by the College of Agricultural and Life Sciences, University of Wisconsin.

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