Functional identification of ATP-driven Ca^{2+} pump in the peribacteroid membrane of broad bean root nodules

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Abstract A Ca^{2+} indicator arsenazo III was used to demonstrate calcium uptake activity of symbiosomes and the peribacteroid membrane (PBM) vesicles isolated from broad bean root nodules and placed in the medium containing ATP and Mg^{2+} ions. This process was shown to be rapidly stopped by vanadate, completely reversed in the presence of the calcium ionophore A23187 but insensitive to agents abolishing electrical potential or pH difference across the PBM. The presence of an endogenous calcium pool within isolated symbiosomes and bacteroids was detected using a Ca^{2+} indicator chlortetracycline. These results prove a primary active transport of Ca^{2+} through the PBM of legume root nodules and provide the first functional identification of an ATP-driven Ca^{2+}-pump, most likely Mg^{2+}-dependent Ca^{2+}-translocating ATPase, in this membrane.

Key words: ATP-dependent Ca^{2+} transport; Ca^{2+}-ATPase; Symbiosome; Peribacteroid membrane; *Vicia faba* L.

1. Introduction

At present it is well known that in plant cells Ca^{2+} is involved as a second messenger in regulating various physiological functions, and its cytosolic free concentration is normally maintained at a sufficiently low level as a result of sequestering an excessive amount of this cation inside intracellular Ca^{2+}-accumulating organelles or its removal from the cells across the plasmalemma [1]. According to the available data, this is likely to be true also for infected nitrogen-fixing cells of legume root nodules. Indeed, recent reports [2–5], indicate that calcium ions are able to control ion and metabolite transport across the peribacteroid membrane (PBM) of symbiosomes, specialized compartments in infected cells containing bacteroids where nitrogen fixation takes place. In particular, it has been established that calcium is required for activity of the protein kinase which, in the soybean at least, stimulates malate uptake by symbiosomes [2,3,6]. This cation also inhibits ion movement through the ammonium channel in the PBM on the bacteroid side in soybean nodules [4,5], and as shown previously by us [7], the transport activity of the PBM H^+-ATPase from yellow lupin root nodules on the plant cytosolic side. Moreover, the data indicating the high calcium content of the bacteroids [4,8,9] suggest that symbiosomes may behave as calcium stores in infected cells.

While all of these findings argue for an important role for calcium in the functioning of symbiosomes, until now Ca^{2+} transport systems in the PBM responsible for sequestering this cation in these nitrogen-fixing units remain unknown.

In the present work, based on the use of the metallochromic Ca^{2+} indicator arsenazo III, we have found, and partly characterized for the first time, the ATP-driven uptake of calcium by purified symbiosomes and by the PBM vesicles isolated from bean root nodules. The data obtained provide evidence for the functioning in the PBM of Mg^{2+}-dependent Ca^{2+}-pumping ATPase, responsible for the active transport of calcium across the PBM. In addition, the existence of the putative Ca^{2+} pool within symbiosomes and bacteroids isolated from bean root nodules has been established in the experiments with the use of the Ca^{2+} indicator chlortetracycline (CTC).

2. Materials and methods

2.1. Chemicals

Sorbitol was obtained from Calbiochem. ATP disodium salt, BTP, HEPES and valinomycin were provided by Sigma. Arsenazo III and CTC were purchased from Serva and Fluka, respectively. All other chemicals were of the highest quality available.

2.2. Plant material and preparation of symbiosomes, the PBM and bacteroid fractions

Broad beans (*Vicia faba* L., cv. Russkie chernye) were grown at 25°C and 70% relative humidity under a 16 h photoperiod on modified nitrogen-free Knop solution. Plants were inoculated with an effective strain 419 of *Rhizobium leguminosarum*, purchased from the Research Institute for Agricultural Microbiology (St. Petersburg, Pushkin). Nodules were harvested when the plants were 5 weeks old (plant flowering stage).

The preparation of symbiosomes, the PBM and bacteroid fractions was performed using the same procedures as those previously described for obtaining such preparations from yellow lupin root nodules [7].

2.3. Ca^{2+} uptake studies

Ca^{2+} uptake by symbiosomes and the PBM vesicles was recorded by continuously monitoring differential (685–650 nm) absorption changes undergone by the metallochromic Ca^{2+} indicator arsenazo III [10], and added to the assay mixture. The reaction was carried out in an unstirred 1 cm light-path cuvette of a Hitachi-557 double wavelength spectrophotometer at room temperature (20–22°C). In most cases the standard assay medium (2 ml) contained 0.4 M sorbitol, 20 mM HEPES-BTP, pH 7.3, 3 mM $MgSO_4$, 27 μM arsenazo III and about 100 μg of total protein. All the experiments were performed in the absence of added calcium in the assay medium which thus merely contained contaminating calcium originating from the chemicals and the samples.

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Abbreviations: PBM, peribacteroid membrane; ATP, adenosine-5′-triphosphate; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CTC, chlortetracycline; BTP, 1,3-bis-(tris(hydroxymethyl)methylamino)propane; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MES, 2-[*N*-morpholino]ethanesulfonic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; oxonol YI, bis93-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol; Δ_ψ, membrane potential; ΔpH, transmembrane pH difference

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2.4. Detection of membrane potential on the PBM of symbiosomes and of PBM vesicles

Formation of an electrical potential across the PBM of intact symbiosomes or of PBM vesicles was detected by measuring the absorbance change of oxonol YI [11], at room temperature with a Hitachi-557 spectrophotometer operated in a dual wavelength mode, sample wavelength at 590 nm and reference wavelength at 610 nm. The assay medium contained 0.4 M sorbitol, 20 mM MES-BTP, pH 6.0, 3 μM oxonol YI and about 30 μg of total protein.

2.5. Detection of Ca^{2+} pool within isolated symbiosomes and bacteroids

A putative calcium pool within isolated symbiosomes and bacteroids was recorded following the fluorescence change of chlortetracycline [12]. A 30 μl sample of symbiosomes or bacteroids was diluted in 950 μl of standard assay medium from which Ca^{2+} was deleted and EGTA (1 mM) sufficient to chelate any external Ca^{2+} present in the sample was included. CTC, 25 μM, was added and fluorescence was measured in a Hitachi-850 fluorescence spectrophotometer set at 380 nm (excitation) and 530 nm (emission).

2.6. Protein

Protein was measured essentially according to the method of Bradford [13], after treatment of the samples with 0.05% Triton X-100 and using bovine serum albumin as a standard.

3. Results

Our initial calcium uptake experiments were performed on purified symbiosomes representing relatively intact nitrogen-fixing units with the PBM in its native orientation. To assess the symbiosome integrity, we conducted control experiments in which the known capacity of symbiosomes to generate membrane potential on the PBM in the presence of ATP and Mg^{2+} ions in the incubation mixture due to the activity of the PBM H^+-ATPase [14], was examined. Fig. 1A shows that isolated symbiosomes exhibited clearly pronounced ATP-dependent electrogenic activity that was inhibited by the addition of vanadate, a well-known inhibitor of P-type ATPases, and rapidly abolished in the presence of the membrane permeable NO_3^- anions.

The ATP-dependent uptake of calcium by symbiosomes suspended in the incubation mixture containing arsenazo III in the presence of Mg^{2+} ions but in the absence of added calcium is shown in Fig. 1B (trace 1). It can be seen that the addition of ATP to symbiosomes initiates a relatively rapid absorbance change of the Ca^{2+} indicator (trace 1) suggesting a removal of Ca^{2+} from the incubation mixture. This response exhibited profound saturable behavior, achieving almost a steady value in about 6–8 min after the addition of ATP and was completely reversed by the subsequent addition of the Ca^{2+} ionophore A23187. A qualitatively different response of the Ca^{2+} indicator insensitive to this ionophore was observed under the same experimental conditions but in the absence of Mg^{2+} ions in the incubation mixture (Fig. 1B, trace 2).

As follows from Fig. 1B, an initial rapid upward deflection of the Ca^{2+} level has been observed upon the addition of ATP. This corresponds to the artificial response of the Ca^{2+} indicator induced by the ATP addition. The essential point is that this artificial response is complete within the mixing time and does not interfere with the kinetics of Ca^{2+} uptake.

Fig. 1B (trace 3) also shows that the MgATP-induced Ca^{2+} uptake by symbiosomes is rapidly stopped by the addition of vanadate and occurs when the assay medium contains both nitrate anions and ammonium cations. These findings demonstrate that the kinetics of Ca^{2+} uptake is not affected by a transmembrane pH gradient or an electrical potential difference which would be abolished by these compounds, respectively. Such a conclusion is confirmed by other data (not shown) indicating that FCCP, nigericin or valinomycin in the presence of potassium ions had no marked effect on the kinetics of the process under study.

Qualitatively very similar results were obtained in the experiments with isolated PBMs (Fig. 2). These experiments were carried out under the same experimental conditions as in the case of symbiosomes. The PBMs as well as symbio-

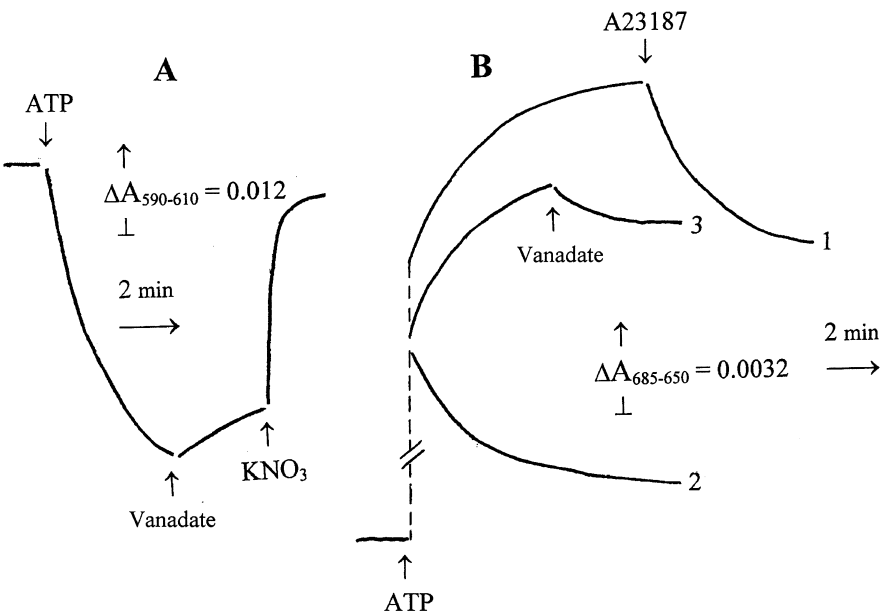

Fig. 1. MgATP-induced generation of membrane potential on the PBM (A) and Ca^{2+} uptake (B) by symbiosomes. At indicated times 1 mM Na_2ATP, 100 μM (A) and 300 μM (B) vanadate, 20 mM KNO_3 and 2 μM A23187 were added. In the case of trace 2 magnesium sulfate was deleted from the standard assay medium, whereas in the case of trace 3 this medium was supplemented with 30 mM KNO_3 and 3 mM $(NH_4)_2SO_4$. Other conditions are described in Section 2.

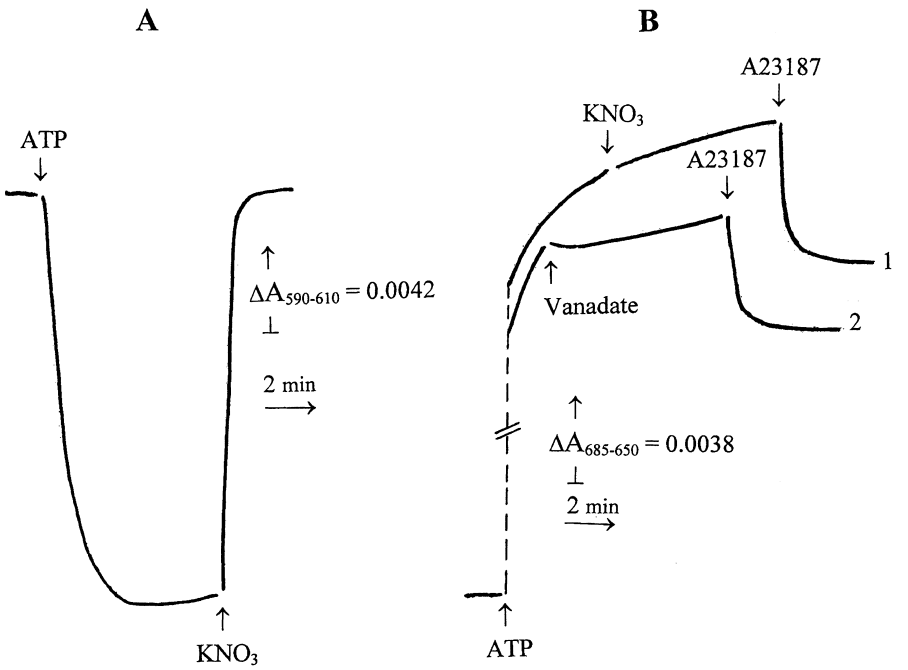

Fig. 2. MgATP-induced generation of membrane potential on the PBM (A) and Ca^{2+} uptake (B) by the PBM vesicles. At indicated times 1 mM Na_2ATP, 20 mM KNO_3, 200 μM vanadate and 2 μM A23187 were added. Trace 1 has been corrected for dilution artefact due to the addition of potassium nitrate. Other conditions are described in Section 2.

somes exhibited pronounced MgATP-induced electrogenic activity that was rapidly abolished in the presence of nitrate anions (Fig. 2A). This demonstrates that the obtained PBM fraction contains sealed right-side-out PBM vesicles.

Some characteristics of the MgATP-induced absorption signal of the Ca^{2+} indicator in the PBM vesicles are presented in Fig. 2B. These characteristics indicate that such a response is sensitive to vanadate and due to the accumulation of Ca^{2+} inside the vesicles. As follows from Fig. 2B (trace 1), nitrate anions had no affect on the observed signal of arsenazo III. This signal also remained essentially unaltered in the presence of ammonium ions in the incubation mixture (not shown). Thus these latter observations again exclude a role for membrane potential or transmembrane pH gradient in driving Ca^{2+} in the lumen of the vesicles.

The Ca^{2+} uptake experiments described above were done at

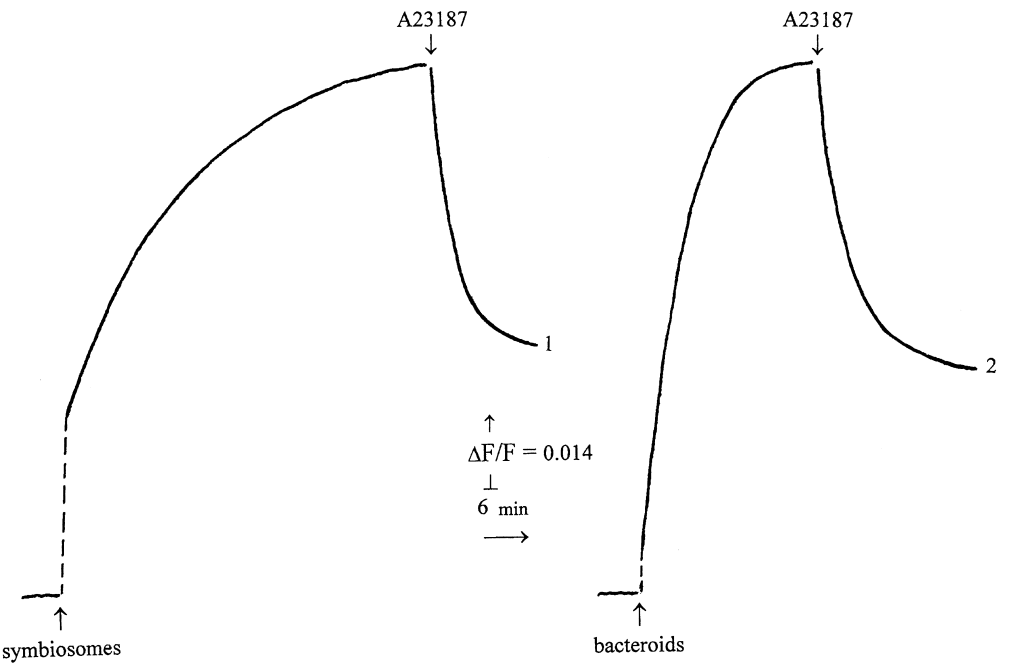

Fig. 3. Kinetics of passive CTC equilibration with the entrapped calcium within isolated symbiosomes (1) and bacteroids (2). Symbiosomes and bacteroids at about 200 μg of total protein were added to the standard assay medium containing 1 mM EGTA and 25 μM CTC. In both cases reversal of the CTC-Ca fluorescence signal was initiated by the addition of 2 μM A23187.

pH 7.3, i.e. in the pH range where the activity of the known Ca^{2+}-pumping ATPases of higher plants achieves maximal values [15]. At the same time, our preliminary data indicated that both the initial rate and the extent of absorbance change of the Ca^{2+} indicator markedly decreased (about 2–3-fold) at both lower (6.0) and higher (8.0) pHs. Therefore it is to be expected that the pH range selected for these studies is favorable for a high calcium uptake activity of symbiosomes and the PBM vesicles.

The above results are in accordance with the available data cited above, suggesting that symbiosomes behave as Ca^{2+}-sequestering compartments in infected cells. In order to test this suggestion in the subsequent experiments we attempted to detect directly the Ca^{2+} pool within both isolated symbiosomes and bacteroids prepared from them, monitoring the fluorescence change of CTC, and the membrane permeable Ca^{2+} indicator [12]. Fig. 3 shows a gradual fluorescence increase resulting from passive CTC equilibration with the entrapped calcium within symbiosomes (trace 1), and bacteroids (trace 2). It can be seen that symbiosomes exhibited a much more slower rate for this process as compared with that in bacteroids. This most likely reflects the fact that bacteroids in symbiosomes are separated from the external medium by two additional barriers for diffusion of CTC: the PBM and the peribacteroid space. However, in both cases the fluorescence signal was to a great, about the same, extent reversed by the subsequent addition of 2 μM A23187. In addition, Fig. 3 shows that in the case of bacteroids the maximal amplitude of the fluorescence signal is very close to that in symbiosomes. Since bacteroids were added to the assay medium at about the same protein concentration as symbiosomes, it can be concluded that the main part of the Ca^{2+} pool within symbiosomes is associated with bacteroids.

4. Discussion

As already noted above, there are several lines of evidence that calcium is of particular importance in nitrogen-fixing infected plant cells, and bacteroids in symbiosomes need to be supplied with this cation in functioning nodules. The only way for such a supplement is to take up calcium from the host cell cytosol. To our knowledge, the results reported in the present work provide the first functional identification of the PBM calcium transporter responsible for calcium transfer from the plant host cell to the microsymbiont, through this membrane.

As established here, the observed Ca^{2+} uptake by symbiosomes and the PBM vesicles requires the presence in the incubation mixture of both ATP and Mg^{2+} ions and is inhibited by vanadate. Therefore, it may be concluded that ATP hydrolysis is involved in this process and most likely catalyzed by the enzyme belonging to P-type ATPases.

The revealed insensitivity of the Ca^{2+} uptake by the preparations obtained to the agents that abolish Δ_ψ and/or ΔpH on the PBM suggests that the putative ATP-driven Ca^{2+} pump directly utilizes the energy of the hydrolysis of ATP for transmembrane Ca^{2+} transport. In other words, this pump acts as the primary energized transport system translocating Ca^{2+} ions electroneutrally across the PBM. In this connection it is important to stress that the activity of the Ca^{2+} pump in the PBM cannot be mediated by that of the H^+-ATPase associated with the same membrane, because the Ca^{2+} uptake was also detected under conditions excluding the formation of the $\Delta\mu_{H^+}$ by the PBM H^+-ATPase.

The data considered above are consistent with other results obtained indicating the presence of the Ca^{2+} pool inside isolated symbiosomes or bacteroids. This pool was detected by the increase in fluorescence of CTC, the Ca^{2+} indicator that is known to monitor sequestered calcium in the presence of membranes in the concentration range 0.1–30 mM [12]. Therefore, judging by the significant increase in CTC-Ca fluorescence intensity after the addition of both symbiosomes and bacteroids to the assay medium, the size of the Ca^{2+} pool within these structures may be substantial. As noted above, most of the calcium in symbiosomes is obviously accumulated inside bacteroids. On the other hand, the observed A23187-induced calcium efflux from MgATP-energized symbiosomes (Fig. 1B, trace 1) suggests that this cation is accumulated in the peribacteroid space as well. However, the precise pattern of calcium compartmentation within symbiosomes is still to be defined.

Although the physiological significance of the capacity of symbiosomes to take up calcium ions is at this time unclear, these nitrogen-fixing units may have an important role in regulating the cytoplasmic calcium level in infected nodule cells and thus functionally replace vacuoles in such cells, in accordance with the idea put forward by Mellor [16]. At the same time, Ca^{2+} involvement in the processes also occurring within symbiosomes or bacteroids during symbiotic nitrogen fixation cannot be excluded.

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References

[1] Bush, D.S. (1995) Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 95–122.
[2] Bassarab, S. and Werner, D. (1987) J. Plant Physiol. 130, 233–241.
[3] Ou Yang, L.-J., Whelan, J., Weaver, C.D., Roberts, D.M. and Day, D.A. (1991) FEBS Lett. 293, 188–190.
[4] Tyerman, S.D., Whitehead, L.F. and Day, D.A. (1995) Nature 378, 629–632.
[5] Whitehead, L.F., Tyerman, S.D., Salom, C.L. and Day, D.A. (1995) Symbiosis 19, 141–154.
[6] Weaver, C.D., Crombie, B., Stacey, G. and Roberts, D.M. (1991) Plant Physiol. 95, 222–227.
[7] Andreev, I., Dubrovo, P., Krylova, V., Andreeva, I.N., Koren'kov, V., Sorokin, E.M. and Izmailov, S.F. (1997) J. Plant Physiol. 151, 563–569.
[8] Vincent, J.M. and Humphrey, B.A. (1963) Nature 199, 149–151.
[9] Andreeva, I.N., Kozharinova, G.M. and Izmailov, S.F. (1995) Dokl. Akad. Nauk. 344, 402–406.
[10] Scarpa, A. (1979) Methods Enzymol. 56, 301–337.
[11] Apell, H.-J. and Bersch, B. (1987) Biochim. Biophys. Acta 903, 480–494.
[12] Dixon, D., Brandt, N. and Haynes, D.H. (1984) J. Biol. Chem. 259, 13737–13741.
[13] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[14] Udvardi, M.K. and Day, D.A. (1989) Plant Physiol. 90, 982–987.
[15] Evans, D.E. and Williams, L.E. (1998) Biochim. Biophys. Acta 1376, 1–25.
[16] Mellor, R.B. (1989) J. Exp. Bot. 40, 831–839.