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Identification and isolation of ATP transport protein in mycobacillin sensitive Aspergillus niger

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Abstract. The temperature sensitive release and uptake of ATP through the *Aspergillus niger* G_3Br membrane vesicles followed saturation kinetics. Both the processes which occurred in the absence of mycobacillin were greatly enhanced by its presence. Liposomes prepared with antifilipin sterol and lipid showed the release and uptake of ATP in the presence of filipin, but no such uptake and release was seen with antimycobacillin sterol and lipid in the presence of mycobacillin. However the liposomes supplemented with *Aspergillus niger* membranes protein (s) showed the release and uptake of ATP, implicating membrane protein as a carrier in the transport process.

Keywords. Membrane vesicles; liposome; reconstituted liposome; isolation of ATP transport protein; uptake and release of ATP.

Introduction

It is generally believed that cell membranes are impermeable to ATP (Glynn, 1968) although there are indications in the literature that ATP might be able to cross the cell membrane. Weideman *et al.* (1969) suggested that rat kidney cortical cells are permeable to ATP. Chaudry and Gould (1970) reported the entry of ATP into intact skeletal muscles, liver and kidney cells and further suggested that this entry might be a carrier-mediated process (Chaudry *et al.,* 1976). Release of ATP from motor nerve terminals on indirect stimulation of the mammalian nerve-muscle preparation was observed by Silinsky and Hubbard (1973). The position regarding the release and uptake of ATP is however very unclear for prokaryotic system. Previous work from our laboratory has shown that mycobacillin (Majumdar and Bose, 1958) caused enhanced release as well as uptake of ATP along with other cell constituents in case of the sensitive strain *Aspergillus niger* G_3Br (Das *et al.,* 1986a) which we suggested to be a carrier mediated process (Das *et al.,* 1986b). In view of this we further studied using membrane vesicles to show that the release and uptake of ATP might implicate a protein. This putative protein was isolated from the *A. niger* membrane vesicles and incorporated into liposome to identify that it is a ATP transport protein.

Materials and methods

Mycobacillin was prepared from the culture filtrate of *Bacillus subtilis* B_3 by the method of Majumdar and Bose (1960). Helix pomatia was purchased from L′ Industrie Biologique Francaise Gennevlliers, France. Filipin was kindly provided by

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Abbreviation used: DCP, Dicetyl phosphate.

Dr. G. B. Whitfield, Upjohn Company, Kalamazoo, Michigan, USA. RNase, Dnase glutathion, lecithin, cholesterol, dicetyl phosphate (DCP), EDTA, DDT, and ATP were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. [^{3}H]ATP was purchased from Bhabha Atomic Research Centre, Bombay. All other chemicals used were of reagent grade and obtained from commercial sources.

Microorganism

A sensitive strain of *A. niger* was used through out the experiments. Mycelial growth (as spherules) of log phase cells (2 days old) grown in Czapek broth at 32 ± 1°C with shaking was used.

Preparation of membrane vesicles from the sensitive strain of A. niger

Membrane vesicles from the sensitive organism *A. niger* were prepared from the protoplasts of *A. niger* by the method of Kaback (1974). Purity of the membrane fraction was checked by Chit in synthetase (Duran *et al.*, 1975).

Preparation of depleted membrane vesicles

Depleted membrane vesicles were prepared with the addition of 50 mM Tris/maleate buffer, pH 7 at a concentration of 10 mg of membrane protein/ml of buffer and incubated for 90 min with gentle shaking at 37°C. The amount of membrane concentration was measured in terms of their protein content.

ATP uptake by depleted and release from preloaded membrane vesicles

ATP uptake by depleted membrane vesicles was studied according to the method of Chaudry and Baue (1980). The reaction mixture (1 ml) contained 4 mg membrane protein, 100 mM Tris/maleate buffer (pH 7), 100 μl of mycobacillin (250 μg/ml), 10 mM ATP containing 2 μCi of [^{3}H]ATP specific radioactivity 2500 Ci/mol. In order to perform ATP release experiments, membrane vesicles were depleted first and then preloaded in 100 mM Tris/maleate buffer, pH 7, containing 10 mM non-labelled ATP and 2 μCi of [^{3}H]ATP specific radioactivity 2500 Ci/mol and incubated with gentle shaking at 37°C for 2 h. At the end of incubation the membrane suspension was separated by rapid centrifugation. Uptake and release of ATP were expressed as the amount of substrate incorporated in nmol/mg of membrane protein being calculated from the observed radioactivity (cpm) per mg membrane protein and the specific radioactivity *i.e.*, cpm per nmol of the substrate.

Recovery of membrane protein from membrane vesicles

For reconstitution studies the membranes were solubilized by 1% Triton X-100. The solubilized membrane proteins were precipitated by the addition of solid $(NH_4)_2SO_4$ to 80% saturation and the precipitate washed twice to remove adhering

detergent. Dialysis buffer (50 mM phosphate buffer pH 6·8) was used to free $(NH_4)_2 SO_4$ and detergent if any from protein suspension.

Preparation of liposomes and incorporation of membrane protein in its lipid bilayer

Liposomes were prepared with a mixture of phospholipid/cholesterol/DCP in a molar ratio 7 : 2 : 1 according to the method of Gregoriadis and Ryman (1972). During the preparation of liposome, antimycobacillin sterol (cholesterol) and lipid (lecithin) were used (Halder and Bose, 1971, 1973). The resulting liposomes were incubated with different concentrations of membrane protein separately for 30 min at room temperature containing 10 mM $MgCl_2$. The liposomes with entrapped ATP were separated from the unentrapped material by centrifugation at 105,000 *g* and repeated washing in buffer.

Results

Kinetics of uptake and release of ATP through membrane vesicles in the presence and absence of mycobacillin showed a linear increase with time till they attained the peak values (figure 1A). The presence of mycobacillin hastened the uptake as well as the release process. The temperature profiles of the kinetics of uptake and release of ATP through membrane vesicles under the above specified condition, further showed that both the processes were temperature sensitive, being adversely affected by high temperature (figure IB).

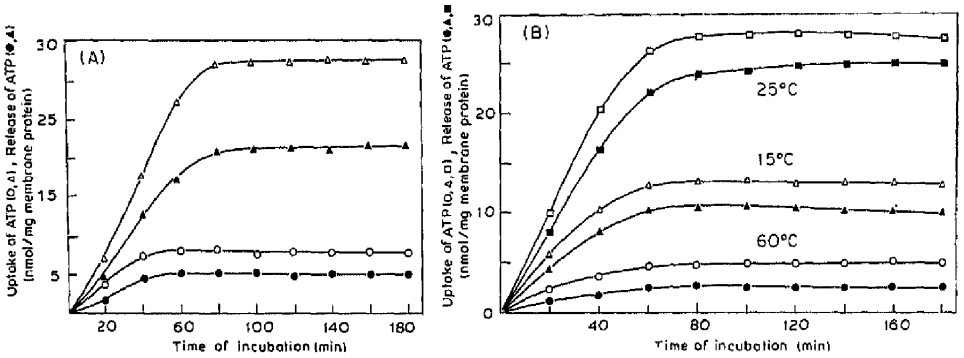

Figure 1. (**A**) Effect of mycobacillin on the kinetics of uptake and release of ATP through membrane vesicles. The process of uptake and release of ATP was followed by the gain or loss of radioactivity by membrane vesicles. (Δ, O). Uptake and (▲, ●) "release of ATP in the presence and absence of mycobacillin. (**B**). Temperature profiles on the kinetics of uptake and release of ATP through membrane vesicles. The reaction mixtures were incubated with shaking at 15°, 25° and 60°C separately in the presence of mycobacillin for different lengths of time.

Uptake and release of ATP through membrane vesicles as a function of ATP concentration in the presence and absence of mycobacillin showed that the uptake as well as release of ATP increased gradually with increase in ATP concentrations till it attained a maximum value. The amount of ATP taken up was 40 nmol in the

presence of antibiotic as against 7·5 nmol in its absence suggesting the stimulatory action of the antibiotic (figure 2).

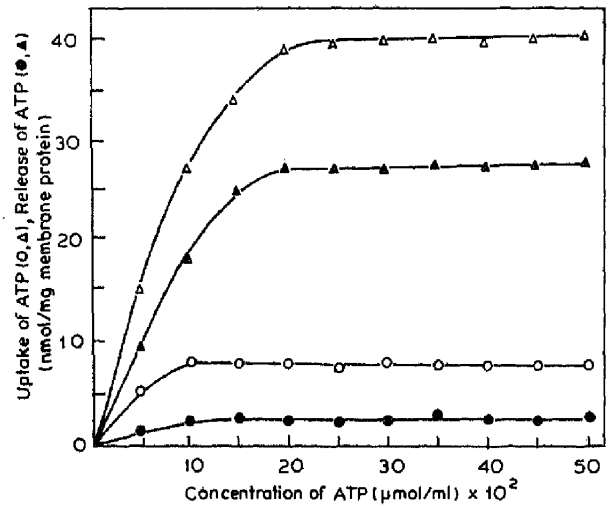

Figure 2. Effect of different concentrations of substrate (ATP) on uptake and release through membrane vesicles. Uptake was followed by depleted and release by preloaded membrane vesicles in the presence of varying concentrations of ATP. (Δ, ○). Uptake and (▲, ●) release of ATP in the presence and absence of mycobacillin.

Effect of different concentrations of mycobacillin on the uptake and release of ATP through membrane vesicles in the presence of an optimum concentration of ATP showed that release recorded a linear increase with increasing concentrations of mycobacillin till it become constant at 150 μg/ml of antibiotic. Uptake also recorded a linear increase up to 25 μg/ml beyond which a rapid decline was observed due to simultaneous release of accumulated ATP caused by mycobacillin (figure 3).

Table 1 indicates that mycobacillin had no effect on the release or uptake of ATP by liposome. However filipin could cause release (65%) and uptake (72%) of ATP from this liposome without being supplemented with membrane protein. Experiments relating the effect of mycobacillin on the uptake and release of ATP from liposome supplemented with membrane protein(s) showed that both uptake and release of ATP from liposome in which membrane protein(s) were incorporated in its lipid bilayer was increased with increasing concentrations of membrane protein in the presence of mycobacillin (data not shown).

Discussion

In continuation of our previous work (Das *et al.,* 1986a, b, 1987) which showed the release and uptake of ATP by the whole cells of a sensitive strain of *A. niger* to be a carrier mediated process, we carried out the study with membrane vesicles and liposomes instead of whole cells to provide additional evidence. These studies showed that release and uptake of ATP by membrane vesicle were enhanced by

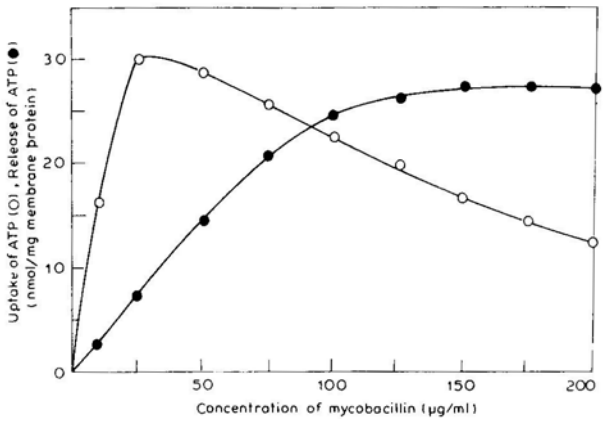

Figure 3. Effect of different concentrations of mycobacillin on the uptake and release of ATP through membrane vesicles. The effect of mycobacillin concentrations on uptake (O) and release (●) of ATP was studied under conditions (controlling parameter, peak concentration of ATP from figure 2, and maximum time incubation from figure 1) of maximum uptake and release.

Table 1. Uptake and release of ATP from liposome in the presence of mycobacillin, filipin and detergent t. Triton X-100.

Release of entrapped [^{3}H] ATP			Uptake of [^{3}H] ATP by liposome		
System	Radioactivity in liposomes at the end of incubation period	Release (%)	System	Radioactivity in liposomes at the end of incubation period	Uptake (%)
Liposome with entrapped [^{3}H] ATP + mycobacillin	1735	2·52	Liposome + [^{3}H] − ATP + mycobacillin	120	1·76
Liposome with entrapped [^{3}H] ATP + filipin	620	65·1	Liposome + [^{3}H] − ATP + filipin	4878	71·8
Liposome with entrapped [^{3}H] ATP + Triton X-100	75	95·7	Liposome + [^{3}H] − ATP + Triton X-100	175	2·5

Uptake and release of ATP was followed under conditions as stated in the text. For the study of release, [^{3}H] ATP was entrapped into the liposome whose initial radioactivity was 1,780 cpm. For the study of uptake, [^{3}H] ATP was added to the suspension buffer whose initial radioactivity was 6790 cpm. Concentrations of mycobacillin, filipin and Triton X-100 used were 250 μg/m1, 1 mg/ml and 0·1% (v/v) respectively. The concentration of liposome used was of OD 0·612 at 660 nm.

mycobacillin. The uptake of ATP followed saturation kinetics (figure 2) implicating a carrier in the process which was further supported by the temperature sensitivity of both the processes (figure IB).

The uptake and release were further studied with liposome using antimycobacillin and antifilipin cholesterol and lecithin. These studies showed that mycobacillin did not have any effect on the process whereas filipin caused release of

ATP as usual. The putative membrane protein was thereafter isolated and incorporated into liposome. When liposome was supplemented with membrane protein in its lipid bilayer, the release of ATP occurred as usual in the presence of mycobacillin. This action of mycobacillin which differed from that of filipin might be due to action of mycobacillin on membrane protein in the lipid bilayer of liposome. Previously we reported that mycobacillin action was antagonised by cholesterol and lecithin. Hence the inaction of mycobacillin towards lipid in the absence of protein while its positive action toward liposome supplemented with membrane protein isolated from *A. niger* membrane vesicles, is a clear indication that ATP was transported by a carrier protein and that the enhancing action of mycobacillin might be due to its action on this lipo-protein complex whose conformational change might be responsible for the release and uptake of ATP**.**

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