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Isolation and properties of an ATP transporter from a strain of *Aspergillus niger*

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A purified ATP transporter from *Aspergillus niger* did not show release or uptake for any of the nucleotides (ADP or UTP) except ATP. The release and uptake did not result from non-specific binding, but appeared to be concentration-dependent processes. ATP was shown by a double-isotopic technique to be transported across membrane vesicles without degradation. The ATP-transport protein was purified to near homogeneity from the membrane vesicles of a strain of *A. niger* and its apparent M_r was approximately 60000. The purified protein showed the properties of a membrane-bound protein in that the carrier protein was shown, during the liposome-preparative process, to translocate from the aqueous phase into the lipid bilayer of the liposome, unlike the cytosolic protein glucose-6-phosphate dehydrogenase, which remained confined to the aqueous compartment. Mycobacillin, a lipid-reactive antibiotic, was bound to the transport protein at a site other than the ATP-binding site, leading to its enhanced release or uptake, which was very feeble in absence of the antibiotic.

Keywords: specific transport of intact ATP; isolation of transporters; reconstituted liposome; enhanced transport; lipid-reactive mycobacillin.

While working on the mode of action of mycobacillin on a sensitive strain of *Aspergillus niger* G_3 Br, it was observed that mycobacillin [1] caused enhanced release and uptake of some normally releasable (i.e. released when whole cells were exposed to buffer in the absence of antibiotic) cell constituents (e.g. Pro, Lys, ATP, P_i, Na^+, K^+ and Ca^{2+}) from the *A. niger* G_3 Br, but had no effect on the intracellular pool concentrations of other cell constituents (e.g. Ala, Asp, Mg^{2+} and UTP) [2–4]. This translocation followed saturation kinetics, implicating a transport protein in the process. By means of a fluorescent probe, the membrane was identified as the target site of action of mycobacillin for enhanced release or uptake of specific cell constituents. These studies detected release or uptake of ATP across the membrane [5] in lower eucaryotes, although the presence of such a transporter had been reported in higher eucaryotes: e.g. permeability of rat kidney cortical cells to ATP [6]; release of ATP from active skeletal muscle under conditions in which potassium was not released [7]; release of ATP from motor-nerve terminals on indirect stimulation of the mammalian nerve/muscle preparation [8]; utilization of ATP in the extracellular space in the process of intracellular phosphorylation, being translocated by some mechanism across the squid axon membrane [9]; and accumulation by soleus muscle and hemidiaphragm when incubated *in vitro*, of [α-^{32}P]ATP and [^{14}C]ATP from the external medium [10]. These transport processes follow saturation kinetics, implicating a carrier in the processes [11–13]. In procaryotic systems there are only a few reports on ATP transport. The uptake of ATP by marine bacteria [14] may be cited as an instance. The obligate intracytoplasmic bacterium *Rickettsia prowazekii* has evolved, unlike free-living bacteria, mechanisms to transport many large charged metabolites directly from the host-cell's cytoplasmic pools. These include 5,5-diphosphoglucose [15], NAD [16], AMP [17], ADP and ATP [18]. An obligate transport system specific for ATP/ADP has been identified in *R. prowazekii* [19]. Nucleotide transport across the cytoplasmic membrane of photosynthetic *Rhodobacter capsulatus* is unique [20–22] in that other free-living gram-negative bacteria lack this capacity [23]. Although the outer membrane forms a barrier for nucleotide transport, right-side-out cytoplasmic-membrane vesicles of *R. capsulatus* cells mediate nucleotide transport because of the presence of a nucleotide transporter located in the cytoplasmic membrane [24].

Hence our earlier work on release and uptake of ATP by intact cells [4] was extended to show the temperature-sensitive release or uptake of ATP through membrane vesicles with saturation kinetics. Furthermore liposomes prepared with sterols, and lipids that antagonize filipin showed release and uptake of ATP in presence of filipin. No such release or uptake of ATP in presence of mycobacillin was seen with liposomes prepared with sterols or lipids that can antagonize the action of mycobacillin. However liposomes containing membrane protein show release and uptake of ATP, implicating a protein carrier in the process [25]. In this communication we report on the isolation and physicochemical properties of ATP transporter from *A. niger* G_3 Br.

MATERIALS AND METHODS

Chemicals and radiochemicals. Mycobacillin was prepared from the culture filtrate of *B. subtilis* B_3 by the method of Majumder and Bose [26]. Phospholipids and sterols were prepared from *A. niger* G_3 Br as described [27–29]. *Helix pomatia* extract was purchased from L'Industrie Biologique Francaise. Filipin complex was kindly provided by Dr G. B. Whitfield, Up John Company, Kalamazoo, Michigan. 10 mg/ml dimethyl formamide

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Abbreviation. Glc6*P* dehydrogenase, glucose-6-phosphate dehydrogenase.

was prepared just prior to use. RNase, DNase, dimethyl formamide, glutathione, β-glucuronidase, phospholipids (phosphatidyl choline, sphingomyelin) cholesterol, dicetyl phosphate, EDTA, dithiothreitol, ATP, AMP, UTP, ADP, column materials (Sephadex G-75; carboxymethyl-cellulose) and the calibration proteins (phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, urease, bovine serum albumin, catalase, lactic dehydrogenase and cytochrome *c*) used for M_r determination were obtained from Sigma Chemical Co. [α-^{32}P]ATP (triethylammonium salt) and [8-^{14}C]ATP (tetrasodium salt) were obtained from New England Nuclear Corp. [^{3}H]ATP was purchased from New Bhabha Atomic Research Centre. The homogeneity of tritiated ATP was confirmed by chromatography with an authentic sample. All other chemicals used were of reagent grade and obtained from commercial sources.

Microorganisms. A sensitive strain of *A. niger* G_3 Br was used throughout the experiments. Micelial growth (as spherules) of log-phase cells (2 days old) grown in Czapek broth at 32 ± 1 °C with shaking was used. The average diameter of spherules was measured microscopically and was found to be 2.1 mm.

Preparation of membrane vesicles from *A. niger* G_3 Br. For this purpose, protoplasts of *A. niger* G_3 Br were prepared by the method of Bachmann and Bonner [30]. Membrane vesicles were prepared from the protoplasts of *A. niger* G_3 Br by the method of Bachmann [31], modified by Das et al. [2]. The membrane pellet was fractionated by means of discontinuous-sucrose-density-gradient centrifugation at $100000\times g$ for 15 h at 4°C by the method of Markel et al. [32].

Purity of the membrane fraction was checked by measurement of chitin synthetase [33] and succinate dehydrogenase [34]. For each experiment a fresh preparation of membrane vesicles was taken, as the membrane vesicles loose their activity quickly. The membrane concentration was measured in terms of their protein content. The protein content of the membrane vesicles was measured by the method of Gornall et al. [35].

Preparation of ATP-depleted and of ATP-loaded membrane vesicles of *A. niger.* Depleted membrane vesicles were prepared according to the method of Chowdhury and Bose [25]. For preparation of loaded membrane vesicles, depleted membrane vesicles were suspended in 100 mM Tris/maleate, pH 7.0, containing 10 mM non-labelled ATP and 2.0 µCi [^{3}H]ATP (specific activity 2500 mCi/mmol) and incubated with gentle shaking at 37°C for 2 h.

Specificity of uptake and release of ATP by membrane vesicles. For uptake studies, membrane vesicles (0.4 mg membrane protein/ml) in 50 mM Tris/maleate, pH 7.0, containing 5 mM ATP or ADP or UTP, and 2 µCi [^{3}H]ATP or [^{14}C]UTP (specific radioactivity 2500 Ci/mol, 12.0 Ci/mmol and 300 Ci/mol, respectively) were incubated for 1 h at 37°C with gentle shaking. At the end of incubation the membrane vesicles were harvested and washed with ice-cold buffer until the washings were free of radioactivity. The radioactivities in the pellet and supernatant were measured. For release experiments, loaded vesicles were incubated under similar conditions in the same buffer without nucleotide, and the radioactivity was determined in the same way.

Characterization of the nature of uptake and release as either a translocation or a non-specific-binding process. Membrane vesicles (0.4 mg/ml) were incubated in 50 mM Tris/maleate, pH 7.0, containing 5 mM ATP and 2 µCi [^{3}H]ATP (specific activity 2500 mCi/mmol) for 1 h at 37°C under gentle shaking. After incubation, the loaded vesicles were harvested and washed until they were free of radioactivity. The radioactivities in the pellet and supernatant were measured. The pellet was divided into two parts, one part being incubated in 50 mM Tris/maleate, pH 7.0, without ATP, while the other part was incubated in 50 mM Tris/maleate, pH 7.0, containing 5 mM nonlabelled ATP. The two samples were incubated for 60 min at 37°C with shaking, then centrifuged. The radioactivities of the pellet and supernatant were measured.

Assay of uptake or release of intact ATP through membrane vesicles by the double-isotopic technique. This assay was carried out according to the method of Chaudry and Baue [10] by the double-isotopic technique. ATP was labelled with ^{14}C and ^{32}P. The ^{32}P at the α-position of [8-^{14}C]ATP was selected to verify whether the nucleotide, when transported across the membrane as the monophosphate, diphosphate or triphophate, remained intact, or whether it was broken down and resynthesized. Membrane vesicles (0.4 mg membrane protein/ml) in 50 mM Tris/maleate, pH 7.0, containing 5 mM ATP including 2 µCi [α-^{32}P]ATP (specific radioactivity 12.0 Ci/mmol) and 2 µCi [8-^{14}C]ATP (specific radioactivity 2.8 Ci/mmol) were incubated for 1 h at 37°C with gentle shaking. At the end of the incubation, the membrane vesicles were harvested (with retention of the membrane-vesicle-free supernatant) and washed with ice-cold buffer until the washings were free of radioactivity. Membrane vesicles were solubilized by 1% Triton X-100. Samples (50 µl) of the solubilized membrane vesicles and membrane-free supernatant were applied separately to a Whatman 3 mm paper strip, as were 10-µl aliquots of 5 mM ATP, ADP, AMP and adenosine. After electrophoretic separation by the method described [36], spots were located under an ultraviolet lamp (Hanovia chromatolite with fluorescence filter), cut from the paper and placed in a vial with 5 ml liquid-scintillation fluid. Radioactivity was measured in a Packard scintillation counter.

For release experiments the membrane vesicles obtained after uptake experiments were treated as loaded membrane vesicles. These loaded membrane vesicles were incubated in 50 mM Tris/maleate, pH 7.0, with gentle shaking at 37°C for 1 h. Thereafter the incubation buffer was analyzed cromatographically to show the presence or absence of ATP-degradation products in the same way as in uptake studies.

Method of isolation of ATP-transport protein from *A. niger* membrane vesicles. Membrane fractions (1 g protein), obtained from the sucrose-density gradient, was suspended in 50 mM Tris/HCl, pH 7.4, containing 1% sodium cholate, 5% sodium deoxycholate and 0.5 mM dithiothreitol in 50 ml. The mixture was sonicated in an ice bath for 5 min and centrifuged at $20000\times g$ for 20 min. Solid ammonium sulphate (56 mg/ml) was added slowly to the supernatant with constant stirring. After standing for 20 min, the precipitate was rejected and the supernatant was further treated with solid ammonium sulphate (260 mg/ml). The precipitate obtained at this stage was collected by centrifugation at $20000\times g$ for 30 min. The precipitate was dissolved in 4 ml 50 mM Tris/HCl, pH 7.4, 0.1% Triton X-100. The dissolved fraction was dialyzed against 2 l 50 mM Tris/HCl, pH 7.4, 0.25 mM dithiothreitol for 5 h at room temperature.

The dialyzed enzyme was further purified by incorporation into liposomes followed by recovery of the entrapped protein. Phospholipids (10 mg, obtained from *A. niger*) were suspended in 2 ml 40 mM Tris/HCl, pH 7.4, 2% sodium cholate, 1% sodium deoxycholate and 1 mM dithiothreitol, and sonicated in a Tomy Probe Sonic Oscillator, Model UR-150P, at 20 kHz and 150 W in an ice bath for 5 min. 100 µg protein were added to the phospholipid/detergent mixture (2 ml) and sonicated for 30 s [37]. The mixture was dialyzed against 50 mM Tris/HCl, pH 7.4, 2.5 mM $MgSO_4$, 0.2 mM EDTA and 0.25 mM dithiothreitol for 20 h at 40°C. The reconstituted vesicles were centrifuged at $140000\times g$ for 30 min. The pellet obtained as above was suspended in 0.1% Triton X-100, 0.9% NaCl, 30 mM Tris/HCl, pH 7.4, and 0.5 mM dithiothreitol. The mixture was sonicated in an ice bath for 30 s, then centrifuged at $140000\times g$ for 1 h.

The supernatant was further purified by gel filtration. It was diluted with 2 % Triton X-100 and applied on a Sephadex G-75 column (1.5 cm×60 cm) equilibrated in 25 mM Tris/HCl, pH 7.4, 0.1 % Triton X-100. The column was developed with the same buffer. The fractions were monitored at 280 nm, and protein peaks were pooled separately. Each of the pooled fractions were tested for transport activity. The transporter was further purified on an ion-exchange column, then on a carboxymethyl-cellulose column (1.5 cm×30 cm) equilibrated in 0.001 M Tris/maleate, 0.002 M calcium acetate, pH 6.4, 0.1 % Triton X-100, 0.2 % (by vol.) glycerol. After absorbing the enzyme fraction on the column, the column was washed with 1 l of the same buffer, then proteins were eluted stepwise. The peak fractions were pooled and concentrated to 2 ml. The fractions that contained the transport activity were mixed with sodium cholate to 1 %, and used for electrophoresis on native polyacrylamide gels.

Polyacrylamide-disc-gel electrophoresis. Gels containing 7.5 % (mass/vol.) acrylamide and 0.28 % *N,N'*-methylenebisacrylamide were polymerized in gel tubes to which protein fractions were applied. Electrophoresis was carried out in a glycine/Tris system containing 1.44 % (mass/vol.) glycine and 0.3 % (mass/vol.) Tris/HCl, pH 8.3, at 10°C and 2 mA/tube, until the tracking dye reached the separation gel and at 4 mA/tube thereafter. The gels were stained for protein with Coomassie brilliant blue R 250 and destained in 5 % (by vol.) methanol/ 7.5 % (by vol.) acetic acid [38-40].

For SDS/PAGE gels were polymerized with 0.2 % SDS, and the running buffer used contained 0.2 % SDS and 0.2 % 2-mercaptoethanol. The protein sample was treated with 0.2 % SDS and 0.2 % 2-mercaptoethanol, usually for 15 min at 50°C, then applied to the gel. The gels were run as above, then stained and destained as described [41].

Determination of apparent M_r. *By gel filtration.* The apparent M_r of the ATP transporter was determined by gel filtration [42] on a Sephadex G-75 column (1.5 cm×60 cm). Elution was performed with 25 mM Tris/HCl, pH 7.4, 0.1 % Triton X-100 and 2 % (by vol.) glycerol, and the column was calibrated with phosphorylase *b* (M_r 94000), albummin (67000), ovalbumin (43000), carbonic anhydrase (30000) as marker proteins. The void volume was determined with blue dextran. The distribution coefficient (K_{av}) was determined from $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume of the protein, V_o the void volume and V_t the total volume of the gel bed.

By SDS/PAGE. SDS/PAGE, as described by Weber and Osborn [41], was used for estimation of M_r. Urease (M_r 83000), bovine serum albumin (66000), catalase (58000), lactate dehydrogenase (36000) and cytochrome *c* (11700) were used as the marker proteins.

Assay of ATP-transport activity via proteoliposomes. Different concentration of membrane protein obtained from the different fractions of the column or otherwise were added to phospholipid/detergent mixture and sonicated as described above. A 0.8-ml aliquot of proteoliposomes was filtered on a 25-mm-diameter GSTF filter (Millipore, 0.2-µm pore size) using a chimney with a 9-mm internal diameter [43]. The vacuum pump was turned off, and the vacuum beneath the filter allowed to dissipate. 0.5 ml 50 mM sodium phosphate, pH 7.4, containing 5 mM 2 µCi [^{3}H]ATP (specific radioactivity 2500 Ci/mol) was placed on top of the filter. After 5–6 min, the ATP solution was filtered, and the narrow-diameter chimney was removed and replaced with a normal chimney (16-mm internal diameter). The filter was washed with 5 ml ice-cold 50 mM sodium phosphate, pH 7.4, and radioactivity was measured by liquid scintillation counting. Liposomes not treated with transport protein was used as control.

Table 1. Uptake of intact ATP by depleted vesicles and release from loaded membrane vesicles of *A. niger* G_3 Br. For ATP-uptake experiments, the depleted *A. niger* membrane vesicles (0.4 mg/ml) were incubated for 1 h at 37°C in 2 ml 50 mM Tris/maleate, pH 7.0, and 5 mM ATP including 2 µCi [α-^{32}P]ATP (12.0 Ci/mmol) and 2 µCi [8-^{14}C]ATP (2.8 Ci/mmol). After incubation, membrane vesicles were harvested, solubilized in 2 ml buffer and processed with the supernatant. The radioactivities of the samples were determined as described in the text. For ATP-release experiments, the loaded membrane vesicles (0.8 mg/ml) were suspended in 2 ml buffer, and 1 ml of this suspension was incubated with gentle shaking at 37°C for 1 h. After incubation, membrane vesicles were centrifuged and radioactivity of the supernatant was determined. No radioactive spots other than those of ATP were detected. Membrane vesicles obtained after uptake experiments were treated as loaded membrane vesicles for release experiments.

Sources of ATP	Radioactivity		^{32}P/^{14}C ratio
	^{32}P	^{14}C	
	cpm/50 µl		
Uptake studies			
Extravesicular nucleotides	2350	1605	1.46
Intravesicular nucleotides	1760	1245	1.41
Release studies			
Extravesicular nucleotides	715	500	1.43

Preparation of liposomes containing transport or cytosolic proteins. Sterol and lipid were isolated from *A. niger* as described [27–29]. 0.5 g lipid, 0.2 g sterol and 0.1 g dicetyl phospate were dissolved in $CHCl_3$/MeOH (3:1, by vol.), and a thin dry film consisting of the lipid mixture was prepared under reduced pressure in a rotary evaporator at 37°C [44, 45]. The thin film obtained on the wall of the flask was dispersed by gentle shaking under nitrogen in 5 ml NaCl/P_i (0.05 M sodium phosphate, pH 6.8, 0.85 % NaCl) containing glucose-6-phosphate dehydrogenase (Glc6*P* dehydrogenase) or ATP-transport protein as required. The dispersion was completed by sonication for 1 min at 4°C. The liposomes with entrapped material were separated from the unentrapped material by repeated washing in buffer and ultracentrifugation at 105000×*g* in the cold for 60 min [46].

Recovery of protein from liposome, if incorporated into its aqueous phase. The liposome suspension containing Glc6*P* dehydrogenase or ATP-transport protein was incubated for 90 min with different concentrations of filipin. At the end of the incubation, the suspension was centrifuged. The pellet and the supernatant were assayed for Glc6*P* dehydrogenase and ATP-transport activity.

Recovery of protein from liposomes, if incorporated in its bilayer. For the recovery of protein incorporated into the bilayer, the liposome suspension after incubation was harvested and the pellet was suspended in 0.05 M sodium phosphate, ph 6.8, 0.1 % (by vol.) Triton X-100, to which $(NH_4)_2SO_4$ was added to 80 % saturation to precipitate the protein. The precipitated proteins were separated by centrifugation at 20000×*g* for 30 min, dissolved in NaCl/P_i, and dialyzed against 2 l NaCl/P_i containing 1 % Triton X-100 and 0.25 mM dithiothreitol for 20 h. The dialysate was used as a source of protein.

Determination of protein concentration. The protein concentration was measured by the method of Lowry et al. [47], with bovine serum albumin as the standard.

Table 2. Purification of ATP-transport protein from *A. niger* vesicles. Membrane protein was estimated by Lowry's method [46]. The total transport activity was determined as described in Materials and Methods until it attained a constant value, which gives the total activity against the given amount of protein incorporated into the liposomes.

Purification step	Protein	Total activity $\times 10^3$	Specific activity	Yield	Purification
	mg	nmol	nmol/mg protein	%	-fold
Solubilized membrane	490	2.94	6.0	100	–
40–55% $(NH_4)_2SO_4$ fraction	124	2.67	21.2	91	3.5
Liposome-incorporation technique	22.5	1.98	88.8	67	14.7
Sephadex G-75	3.5	1.44	412.0	49	67.6
Carboxymethyl-cellulose	0.31	0.86	2796.0	29	460

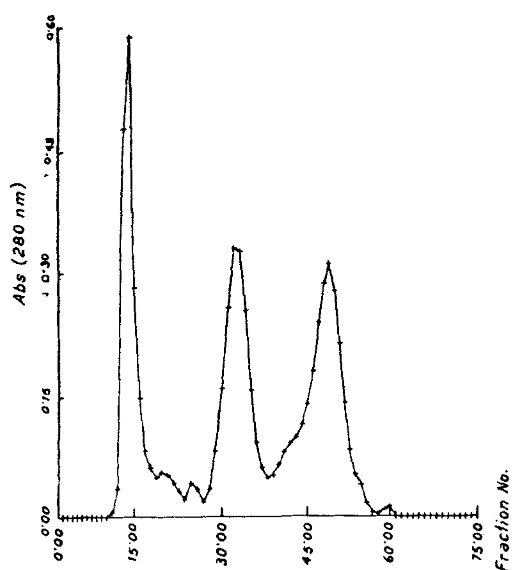

Fig. 1. Sephadex G-75 gel-filtration profile of ATP transporter. The supernatant from the liposome-incorporation technique was applied to a Sephadex G-75 column (1.5 cm×60 cm) equilibrated in 25 mM Tris/HCl, pH 7.4, 0.1 % Triton X-100. The column was then eluted with the same buffer at 15 ml/h. Fractions (5 ml) were monitored by absorbance at 280 nm.

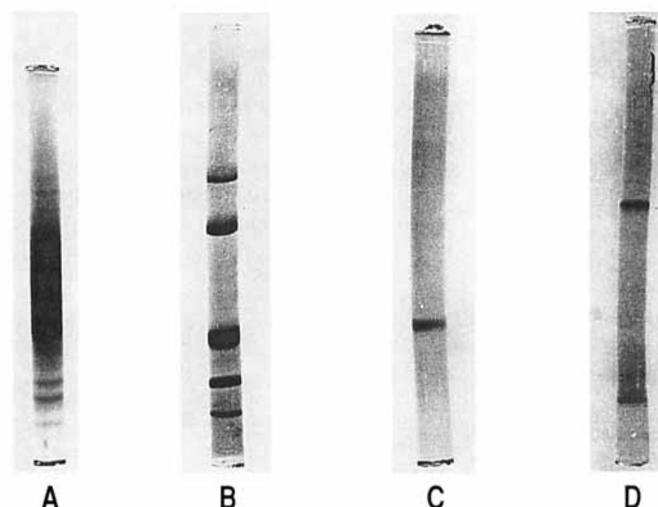

Fig. 2. PAGE of the ATP transporter. PAGE was carried out in a 7.5 % gel containing 0.3 % Triton X-100 at pH 8.3. Electrophoresis was carried out at 4 mA/gel for 6 h with Tris/glycine. 20 µg protein were analyzed after different stages of purification: (A), $(NH_4)_2SO_4$ fractionation; (B), gel filtration (C), carboxymethyl-cellulose chromatography. (D) About 50 µl protein fraction obtained after carboxymethyl-cellulose chromatography was treated with 0.2 % SDS and 0.2 % 2-mercaptoethanol for 15 min at 50°C, then applied to a gel containing 0.2. % SDS.

RESULTS

Experiments with different nucleotides for uptake by depleted vesicles and release from loaded membrane vesicles. The depleted membrane vesicles showed uptake of ATP only and not of ADP or UTP (data not shown). Since release experiments were carried out only with loaded membrane vesicles, release studies were therefore limited to ATP only, which showed considerable release in the ATP-free buffer. The specificity of the transport activity of the ATP transporter was verified with the purest form of the preparation (data not shown).

Characterization of the ATP release as a translocation process distinct from non-specific surface binding. Membrane vesicles were loaded with ATP by incubating in buffer containing 5 mM ATP including 2 µCi [^{3}H]ATP, and incubated in ATP-free buffer, or in buffer containing the same concentration of unlabelled ATP. 50 % release was observed in ATP-free buffer, but negligible (5 %) was observed in the presence of ATP. This differential pattern of ATP release under two conditions strongly suggested that the release might be a concentration-motivated translocation process, as distinct from non-specific binding on the membrane surface.

Transport of intact ATP during uptake by depleted vesicles and release from loaded vesicles. Experiments on the fate of ATP during the uptake process by the depleted vesicles showed that the pellet of membrane vesicles and the membrane-vesicle-free supernatant, as analyzed by chromatography, contained ATP only as added initially and none of its labelled degradation products (ATP being labelled in the α-position), which indicates that ATP was not extravesicularly degraded during the uptake process (Table 1). The $^{32}P/^{14}C$ ratios in ATP from the membrane vesicles and the membrane-vesicle-free supernatant were almost identical, which shows that intact molecules of ATP were taken up by the membrane vesicles.

In the same way experiments on the fate of intact ATP during release from loaded membrane vesicles indicated that membrane vesicles and vesicle-free supernatant contained only ATP and none of its degraded products. Furthermore, the ratios of $^{32}P/^{14}C$ of ATP in the membrane vesicles and vesicle-free supernatant were identical (Table 1), which indicated the release of intact ATP from the loaded membrane vesicle.

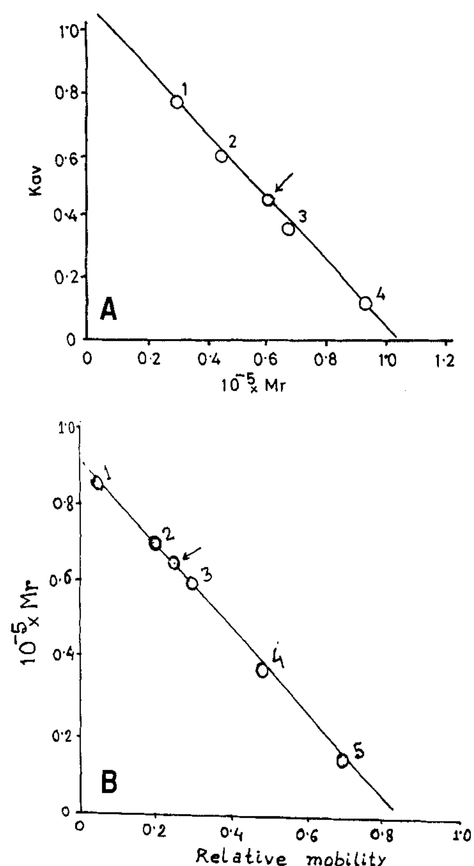

Fig. 3. M_r of ATP transporter. (A) Estimation of the apparent M_r of the ATP transporter by Sephadex G-75 gel filtration. ATP transporter and the marker proteins were applied to the Sephadex G-75 column (1.5 cm×60 cm) equilibrated in 25 mM Tris/HCl, pH 7.4, 0.1 % Triton X-100. The distribution coefficients (K_{av}) for the markers and the ATP transporter were calculated, and the linear correlation between K_{av} and log (M_r) was used to estimate the apparent M_r. The marker proteins were carbonic anhydrase (1), ovalbumin (2), albumin (3), and phosphorylase *b* (4). (B) SDS/PAGE was performed on 5 % acrylamide gels. The marker proteins were urease (1), bovine serum albumin (2), catalase (3), lactate dehydrogenase (4), and cytochrome *c* (5). Their mobilities were plotted against their log (M_r). Arrows indicate the ATP transporter.

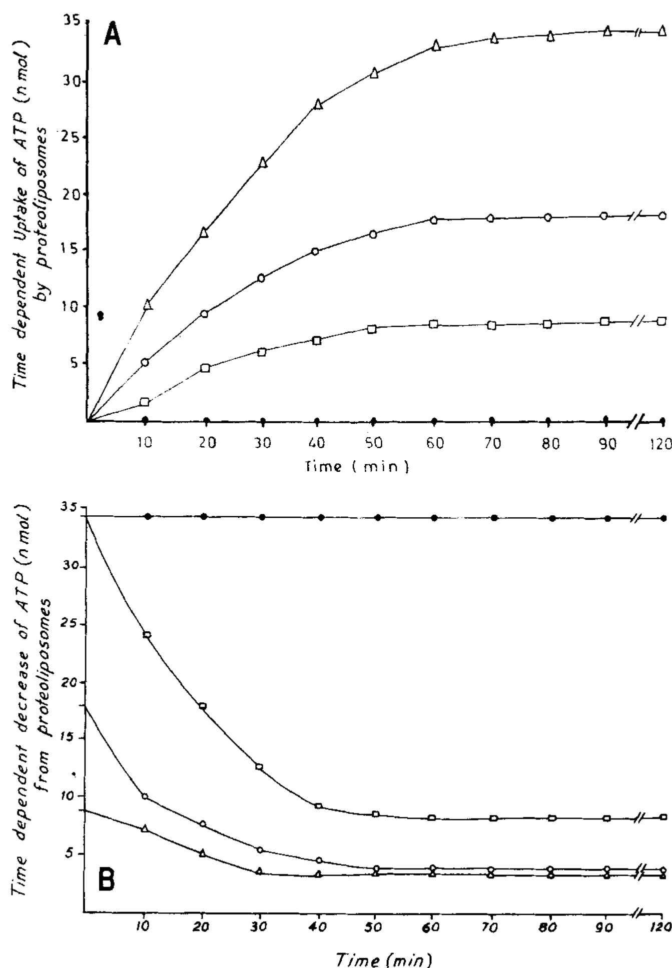

Fig. 4. Kinetics of ATP uptake by (A) and release from (B) liposomes containing transport protein. For kinetic studies, different concentrations of membrane protein were added to phospholipid/detergent mixture and sonicated as detailed in Materials and Methods. The reconstituted liposomes were suspended in 2 ml 50 mM Tris/maleate, pH 7.0, 5 mM ATP containing 2 µCi [^{3}H]ATP (2500 Ci/mol) and incubated at 37°C for different times. As control experiments for uptake, liposomes not containing transport protein were used. For release experiments, liposomes obtained after uptake studies were treated as loaded liposomes. As controls for release experiments, [^{3}H]ATP was entrapped in liposomes not containing ATP-transport protein. □, 10 µg; ○, 30 µg; △, 70 µg protein; ●, control.

Isolation of ATP-transport protein. The ATP carrier was purified as described in Materials and Methods, and the results of typical purification steps are summarized in Table 2. For solubilization of the enzyme activity, several detergents were tried and deoxycholate (0.5 %) was the most effective. The ammonium sulphate fractionation resulted in 3.5-fold purification (as measured in terms of ATP-transport activity) with respect to the crude membrane protein obtained by extraction of the membrane with the detergent mixture. The purification of the transporter, which contains a hydrophobic region, because it is a membrane-bound protein, was attempted by the liposome-incorporation technique. ATP-carrier activity was increased 14.7-fold by the liposome-purification technique with respect to the $(NH_4)_2SO_4$ purification step. The carrier protein was also purified by gel filtration, i.e. on a Sephadex G-75 column (Fig. 1). The fractions collected from the column were monitored by absorbance at 280 nm. Three peaks were obtained. The first fraction showed ATP-transport activity. The ATP-transport activity of this step was increased 67.6-fold in comparison with the liposome-purification step. The protein obtained from gel filtration was applied to a carboxymethyl cellulose column, and only one major peak was obtained (data not shown). It was tested for ATP-transport activity and showed 460-fold purification with respect to the gel-filtration-purified enzyme. The purified materials obtained after four major purification steps, namely $(NH_4)_2SO_4$ fractionation, liposome-incorporation technique, and Sephadex G-75 and carboxymethyl-cellulose chromatographies, were electrophoresed in a 7.5 % polyacrylamide gel (Figs 2 A–C). After $(NH_4)_2SO_4$ precipitation (Fig. 2A, 3.5-fold purification) separation of bands was not observed, whereas after gel filtration (Fig. 2 B; 67.6-fold purification) the separation of bands was observed, and after carboxymethyl-cellulose chromatography (Fig. 2 C) a major sharp band was observed. Thus, the purified transport protein appears to be a single-component protein obtained in four steps with 460-fold purification. The homogeneity of the purified fraction was tested by SDS/PAGE (Fig. 2 D). The purified fraction from carboxymethyl-cellulose column on a 0.2 % SDS/polyacrylamide gel appears to have a single band,

Table 3. Destination of membrane protein and cytosolic protein from the aqueous compartment during liposome preparation. Experiments were performed to locate the site of incorporation of Glc6*P* dehydrogenase or purified protein into liposomes. Liposomes treated with Glc6*P* dehydrogenase or the transport protein were suspended in NaCl/P_i, incubated for 90 min with different concentrations of filipin, and centrifuged. The pellet and the supernatant were assayed for Glc6*P* dehydrogenase and ATP-transport activity. Glc6*P* dehydrogenase activity was measured spectrophotometrically by the rate of formation of NADPH as determined from its absorbance at 340 nm [47]. The amount of Glc6*P* dehydrogenase was obtained from standard calibration curve. The ATP-transport activity in pellets was determined as detailed in the text.

[Filipin]	Glc6*P* dehydrogenase after filipin treatment of liposomes in the		ATP transport activity after filipin treatment of liposomes in the	
	supernatant	pellet	supernatant	pellet
mg/ml	mg		cpm	
1	0.33	0	0	2.480
3	0.45	0	0	2.510

which could not be dissociated into subunits, even by treatment of the protein fraction with 2% SDS and 1% 2-mercaptoethanol for 60 min.

Apparent M_r of the ATP transporter by gel filtration on Sephadex G-75 and by SDS/PAGE. The ATP transporter obtained from the carboxymethyl-cellulose column was chromatographed on the gel-filtration column or run on an SDS/polyacrylamide gel with marker proteins. The distribution coefficient calculated from elution pattern (by gel filtration on Sephadex G-75) was plotted against log (M_r) of the marker proteins Fig. 3A). The M_r of the purified ATP transporter was determined as 60500. The relative mobility of the proteins on SDS/PAGE was plotted against log (M_r) (Fig. 3B), which indicated that the M_r value of purified ATP transporter was 63000.

Kinetics of ATP uptake by and release from liposomes as a function of the amount of transport protein incorporated therein. The rate of uptake, which occurred almost instantaneously, increased progressively with time during the initial period. The rate then attained the maximum values, depending on the protein concentration. Non-amended liposomes did not cause any uptake (Fig. 4A).

Liposomes not containing protein, but loaded with ATP during the preparative process, did not show ATP release, as shown by the absence of any decrease in their ATP content, whereas liposomes showed release when they contained transport protein. Release of ATP content from liposomes increased progressively with the time during initial stages and thereafter the rate of release reached maximum values of ATP release depending on the concentration of transport protein incorporated into liposomes (Fig. 4B). The transport activity (release or uptake) appears to be the same, being 15–25 nmol · min^{-1} · mg $protein^{-1}$.

Destination of cytosolic Glc6*P* dehydrogenase and of the purified ATP-transport protein from the aqueous compartment during liposome preparation. Liposomes prepared with Glc6*P* dehydrogenase were incubated with filipin (1 mg/ml and 3 mg/ml) for 90 min, and centrifuged to give a supernatant and a pellet. The supernatant contained 80% of the Glc6*P* dehydrogenase activity [48], whereas the protein recovered from the pellet with Triton X-100, as detailed in Materials and Methods, did not show any Glc6*P* dehydrogenase activity (Table 3). Similar experiments with lipsomes prepared with purified ATP-transport protein showed that the supernatant did not possess any ATP-transport activity (Table 3), whereas the protein recovered from the pellet contained ATP-transport activity.

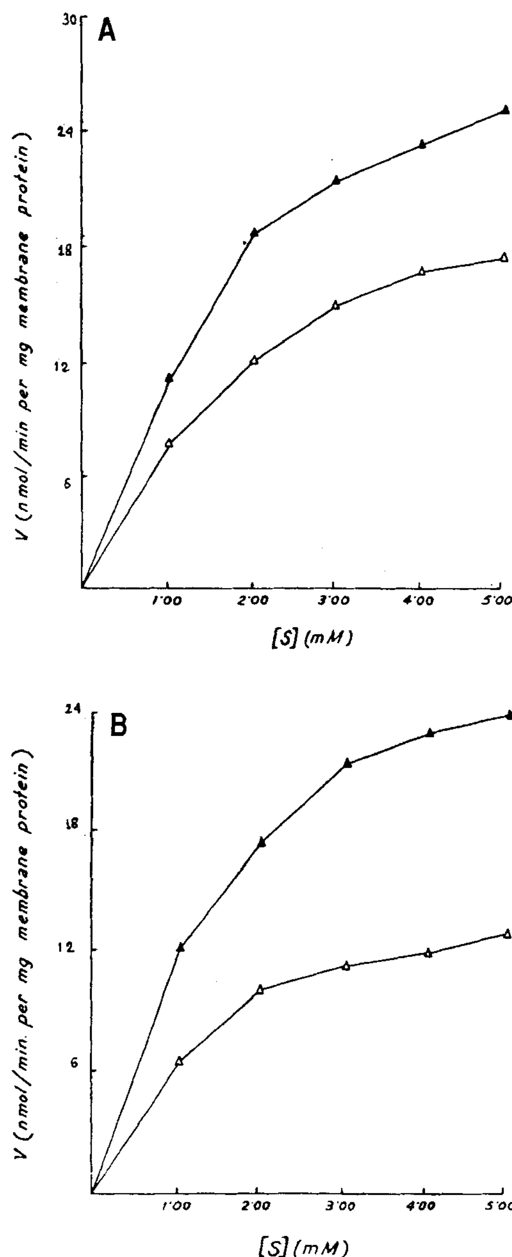

Fig. 5. The rate of uptake (A) and release (B) of ATP via membrane vesicles as functions of the ATP concentrations in the presence and absence of mycobacillin. Membrane vesicles (0.4 mg/ml) were suspended in 50 mM Tris/maleate, pH 7.0, containing ATP in the range of 0–5 mM, and incubated in the absence and in presence of mycobacillin with shaking at 37°C for 1 h. The initial rate was calculated from the linear part of the time course curve. △, no mycobacillin; ▲, 30 µg (A) or 200 µg (B) mycobacillin.

Determination of ATP-binding and mycobacillin-binding sites on the carrier protein from the concentration-dependent uptake process by membrane vesicles. Uptake and release of ATP from *A. niger* membrane vesicles over the range 1.5–2.0 mM ATP was non-linear and followed Michaelis-Menten kinetics in the presence and absence of mycobacillin (Fig. 5).

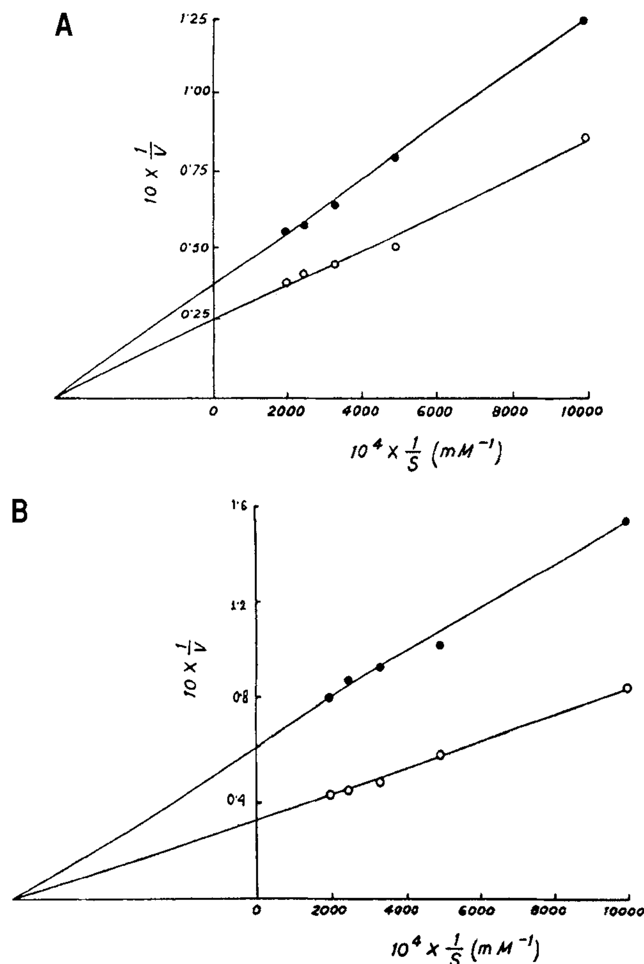

Fig. 6. Comparison of apparent K_m and V_{max} values for the uptake and release of ATP. Values were determined from Lineweaver-Burk plots. Experimental conditions were as in Fig. 5. ●, no mycobacillin; ○, 30 µg (A) or 200 µg (B) mycobacillin.

Table 4. Comparison of apparent K_m and V_{max} values for the release and uptake of ATP in *A. niger* membrane vesicles. Values were determined from Lineweaver-Burk plots.

System	K_m		V_{max}	
	with mycobacillin	without mycobacillin	with mycobacillin	without mycobacillin
	mM		nmol · min^{-1} · mg protein^{-1}	
Uptake	2.40	2.34	39.40	27.31
Release	1.50	1.57	30.08	16.50

However, neither release nor uptake of ATP occurred with heat-treated membrane vesicles. Kinetic constants (Table 4) were calculated from Lineweaver-Burk plots (Fig. 6) for membrane vesicles, which suggests that the apparent K_m values for uptake and release of ATP in the presence and absence of mycobacillin were very much comparable. V_{max} values increased significantly in the presence of mycobacillin for release and uptake of ATP (Table 4). Thus, mycobacillin did not appear to compete with ATP for the ATP-transport protein.

DISCUSSION

In continuation of our earlier work we report here the specificity of the ATP-translocation process by experiments with different nucleotides, which indicated that ATP was the only nucleotide taken up or released during the transport process (data not shown). The uptake and release of ATP have been characterized as a solely concentration-dependent translocation process and do not result from non-specific binding on the surface. This was shown by the differential pattern of release from loaded vesicles: 50% release into the ATP-free buffer but only 5% into buffer containing the same concentration of ATP as used to load the vesicles.

Since ATP is generally degraded during the transport process across the plasma membrane by ATPase activity, experiments involving the double/isotopic technique (with ATP labelled with ^{32}P at the α-position and with ^{14}C at the C8 position) were designed to examine whether intact ATP was taken up or released during the transport process. The results show that translocation of intact ATP molecules occurred without any breakdown or resynthesis during the transport across the membrane (Table 1). Chaudry and Baue [10] applied the double-isotopic technique (ATP labelled with ^{32}P and ^{14}C) to their work on intact rat soleus muscle and hemidiaphragms and made the same conclusion.

We report the purification and properties of the transport protein. The existence of such a carrier protein has been reported earlier in eucaryotic systems [6–12]. In procaryotes, the presence of an ATP-transport protein is not generally reported, although there are reports where such a protein has been shown [14–24].

The apparent M_r of the transporter, which migrates as a single band on SDS/PAGE (Fig. 2) in the purified state is 60500 or 63000, as determined by Sephadex G-75 gel filtration (Fig. 3A) and by SDS/PAGE (Fig. 3B), respectively. These two values are in fair agreement and approximate to 60000. This shows that the ATP transporter may be a monomeric protein, containing a single polypeptide chain, or may be a multimeric one, composed of only one type of subunit.

The purified transport protein was used to study the kinetics of uptake by and release from liposomes containing the transporter. It appears that the kinetics of release or uptake followed the same pattern, reaching the maximum values of ATP translocated, characteristic of the transporter-protein concentrations incorporated into the liposomes. This is what would be expected of a transporter protein in regard to its time-dependent transport activities, provided these activities of the transporter were studied in a closed system, as here. However, in an open system, if so designed as to eliminate the effect of accumulation of ATP translocated and if given sufficient time, the maximum values of ATP transported would have been identical and independent of the transporter concentrations incorporated into the liposomes.

In our kinetic studies on uptake or release, the ATP-concentration gradient (motive force) was approximately kept at 5 mM, while the concentration of the transport protein was varied. This led to variation in the rate of translocation and in the amount translocated, the maximum transport activity being 15–25 nmol · min^{-1} · mg transporter^{-1}. In similar studies by Chaudry and his group [10], using intact rat soleus muscle or hemidiaphragm, the putative transporter was at a constant level, while the concentration of ATP was varied. An increase in ATP from 5 mM to 10 mM led to an increase in the intracellular ATP level in muscles by 75%. The transport activity (intracelluler uptake) was found to be 0.44±0.03 µmol · h^{-1} · g tissue^{-1}. In our studies the transport activity of the membrane vesicles was 25 mmol · min^{-1} · mg protein^{-1}, whereas that by liposomes con-

taining the purified transporter was 15–25 nmol · min^{-1} · mg $protein^{-1}$ and by whole cells was 17 mmol · min^{-1} · mg dry $cells^{-1}$ [4]. It is very difficult to make a comparative study. The transport activity of the purified protein was found to be below that of vesicles or whole cells because of lack of natural environment. The transporter is feebly active in its native conformation, and its activity is greatly enhanced in the presence of lipid-reactive mycobacillin [2–4].

The transport protein translocated from the aqueous phase into the lipid bilayer during liposome preparation, whereas Glc6*P* dehydrogenase, a cytosolic protein, under similar circumstances remained confined to the aqueous phase (Table 3). This finding confirmed that the ATP-transport protein has the usual properties of a membrane transport protein.

Since mycobacillin caused enhanced release and uptake of ATP (Fig. 4), which was feeble in its absence, the uptake process was analyzed by Michaelis-Menten kinetics (Fig. 5), which showed the non-competitive nature of the action of mycobacillin, which might bind the transport protein at a site other than the ATP-binding site (Table 4). This finding and our observation that the enhancing action of mycobacillin on release or uptake was antagonized by cholesterol or lecithin [17] might be taken to mean that the carrier might function in conjunction with some mycobacillin-reactive lipids, whose association (physical or physicochemical) did not cause as much release or uptake in its native form in the absence of mycobacillin, as it did when altered by mycobacillin, being bound at a site other than ATP-binding site.

Thus the protein from *A. niger* membrane vesicles specifically causes translocation of intact ATP across the cytoplasmic membrane and is probably dependent on membrane lipid for its functional activity.

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