# Characterization of the ATP transporter in the reconstituted rough endoplasmic reticulum proteoliposomes

Seung Jin Shin, Woo Kyoung Lee, Hyung Wook Lim, Jong-Sang Park *

*Center for Molecular Catalysis, Department of Chemistry, Seoul National University, Shillim-dong, San 56-1, Kwanak-ku, 151-742 Seoul, South Korea*

## Abstract

Adenosine triphosphate (ATP) transporter from rat liver rough endoplasmic reticulum (RER) was solubilized and reconstituted into phosphatidylcholine liposomes. The RER proteoliposomes, resulting from optimizing some reconstitution parameters, had an apparent $K_m$ value of 1.5 µM and a $V_{max}$ of 286 pmol min$^{-1}$ (mg protein)$^{-1}$ and showed higher affinity for ATP and a lower $V_{max}$ value than intact RER ($K_m$ of 6.5 µM and $V_{max}$ of 1 nmol). ATP transport was time- and temperature-dependent, inhibited by 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, which is known as an inhibitor of anion transporters including ATP transporter, but was not affected by atractyloside, a specific inhibitor of mitochondrial ADP/ATP carrier. The internal and external effects of various nucleotides on the ATP transport were examined. ATP transport was *cis*-inhibited strongly by ADP and weakly by AMP. ADP-preloaded RER proteoliposomes showed a specific increase of ATP transport activity while AMP-preloaded RER proteoliposomes did not show the enhanced overshoot peak in the ATP uptake plot. These results demonstrate the ADP/ATP antiport mechanism of ATP transport in rat liver RER. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* ATP transporter; Reconstitution; Antiport mechanism; Rough endoplasmic reticulum

## 1. Introduction

Adenosine nucleotide, which is an effective energy source of many biological processes, is known to be utilized for several energy-requiring reactions in the lumen of rough endoplasmic reticulum (RER) [1]. These reactions include dissociation of complexes between chaperones and correctly folded and assembled proteins, disulfide formation, and protein polymerization [1–4]. ATP is also the substrate in the lumenal phosphorylation of proteins such as Bip [1] and protein disulfide isomerase [4]. Degradation of misfolded or overexpressed proteins, a process that requires ATP, has been postulated to occur within the lumen of the ER [5,6]. Thus, the existence of ATP transporter of RER to facilitate the entrance of ATP into the lumen has been suggested and the characterization of ATP transport has been reported from mammals and yeast [7,8]. Recently, identification and purification of the protein responsible for the transport of ATP into the lumen of RER have been conducted from *Saccharomyces cerevisiae* and rat liver. Sac1p was identified in *S. cerevisiae* as a protein responsible for ATP transport during protein

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Abbreviations: RER, rough endoplasmic reticulum; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; AAC, ADP/ATP carrier; PMSF, phenylmethanesulfonyl fluoride

* Corresponding author. Fax: +82-2-889-1568;
E-mail: pfjspark@plaza.snu.ac.kr

translocation [9]. In rat liver, a 56-kDa protein, different in size from the Sac1p, was identified as an ATP transporter by photoaffinity labeling and partial purification [10]. Grp 170 from mammalian microsomes was also suggested as a protein mediating efficient insertion of polypeptides into microsomal membrane at the expense of ATP [11].

Since ATP transporters have been reported to exist in other subcellular organelles such as mitochondria [12] and Golgi [13] and have also been found in other cell types such as chromaffin granule [14] and *Aspergillus niger* [15], it is necessary to characterize the mechanism of ATP transport and determine the relation with the other ATP transporters. The ATP/ADP antiport mechanism, a well-known mitochondrial ADP/ATP carrier (AAC) mechanism, was suggested in *S. cerevisiae* [9]. While ATP is transported into the lumen of RER, the change of radiolabeled nucleotide in the medium and lumen was analyzed by HPLC [16]. The ATP transporter in chromaffin granule utilizes GTP and UTP as substrates and accumulates ADP and ATP [14], while the ATP transporter from a strain of *A. niger* takes up ATP as a unique substrate [15].

Various mechanisms of ATP transport have been reported, but direct experimental evidence to support any mechanism of ATP transport has not been presented in mammalian RER. Thus, in this report we show the characteristics of reconstituted RER proteoliposomes with high activity of ATP transport and the evidence of the ATP/ADP antiport mechanism.

## 2. Materials and methods

### 2.1. Materials

Female Wistar rats were obtained from Seoul National University Breeding Laboratories. [8-$^{14}$C]ATP (51 mCi/mmol) was purchased from Amersham Corp. All other chemicals used were purchased by Sigma except Bio-beads SM-2 from Bio-Rad.

### 2.2. Preparation and characterization of RER

RER was obtained from the livers of adult female Wistar rats weighing 200–300 g following the proce-

dure of Fleischer and Kervina [17]. Briefly, rat liver was homogenized in four volumes of microsomal buffer composed of 0.25 M sucrose, 10 mM HEPES, pH 7.4. The homogenate was centrifuged at $960 \times g$ for 10 min to remove nuclear pellets. The supernatant was centrifuged at $12\,000 \times g$ for 30 min to yield a post-mitochondrial supernatant. The post-mitochondrial supernatant was then centrifuged at $105\,000 \times g$ for 1 h in a Beckman 42.1 Ti rotor to yield microsomes, which represented crude endoplasmic reticulum. The microsomes were resuspended in 43% sucrose, 10 mM HEPES, pH 7.4, and overlaid with 38.7, 29, and 8.2% sucrose in 10 mM HEPES buffer (approximate volume ratio was 1:1:1:1) and then centrifuged at $26\,000 \times g$ in a SW 28 rotor for 1 h. The 43% sucrose layer was diluted to 29% sucrose in 10 mM HEPES buffer and 1 M CsCl was added to bring its final concentration to 15 mM. This suspension was layered over a cushion of 1.3 M sucrose in 10 mM HEPES buffer and centrifuged at $237\,000 \times g$ for 110 min in a 90 Ti rotor. The resultant pellet was washed and resuspended in microsomal buffer at a concentration of 5 mg of protein per ml and stored at $-70°C$.

The structural integrity of RER was routinely verified by determination of mannose-6-phosphatase latency [18], which was $>95\%$ in all preparations used. Specific activity of mannose-6-phosphatase in Triton X-100-disrupted RER was $360 \pm 29$ nmol/min per mg of protein ($n = 5$). This amounted to, on average, five times the specific activity found in homogenate ($72 \pm 5$ nmol/min per mg of protein; $n = 5$). Activities of alkaline phosphodiesterase, a plasma membrane marker [19] were also assayed in RER preparation. The microsome, obtained by the centrifugation of the post-mitochondrial supernatant, has the activity of $135 \pm 6$ nmol/min per mg of protein ($n = 5$), while the RER fraction after the discontinuous sucrose gradient showed an activity of 5 nmol/min per mg of protein ($n = 5$).

### 2.3. Solubilization and reconstitution

Extraction of membrane protein was conducted on ice by the resuspension of 15 mg of RER pellet with 1 ml of solubilizing buffer [20 mM HEPES/NaOH pH 7.4, 3% (w/v) Triton X-100, 1 mM MgCl$_2$, 50 mM NaCl, 5% (w/v) glycerol, and 1 mM phenyl-

methanesulfonyl fluoride (PMSF)], where the ratio of protein to detergent was adjusted to 1:2 (w/w). After 30 min, unextracted material was pelleted by centrifugation at 4°C at $105\,000 \times g$ (Beckman 42.1 Ti) for 1 h, and the supernatant was collected and kept on ice.

Egg yolk phosphatidylcholine (PC, 10 mg) in chloroform was dried and lyophilized. The dried lipid was then resuspended in 1 ml of reconstitution buffer [20 mM HEPES/NaOH pH 7.4, 1 mM $MgCl_2$, 50 mM NaCl, and 5% (w/v) glycerol] and sonicated (Sonics and Materials Inc. VCX 600) under nitrogen gas until a clear suspension was obtained. This liposome was usually prepared freshly when used.

Reconstitution was done according to the procedure in which proteins, which have previously had most of detergents removed, are induced to incorporate into preformed liposomes [20]. To remove Triton X-100, the solubilized RER extracts were loaded on 5 ml of Bio-beads SM-2 column and circulated under a flow rate of 0.3 ml/min. Large unilamellar proteoliposomes were made by the freeze–thaw procedure. After 60 min, the column eluate (1 ml) was quickly mixed with liposomes (600 μl) and the mixture, aliquoted to 100 μl, was frozen in liquid $N_2$ and allowed to thaw at room temperature. This cycle was repeated four times. After the fourth thawing, the mixture was sonicated for 15 s and applied to a Sephadex G-50 column ($1 \times 15$, flow rate:0.33 ml/min) and to a Sepharose 4B ($1 \times 100$, flow rate: 0.1 ml/min) column for analysis. Proteoliposomes, where nucleotide was preloaded, were made by using the reconstitution buffer containing 200 μM of nucleotides. Proteoliposomes eluted in the void volume of gel filtration column were used for transport assay. The size of proteoliposomes was determined by transmission electron microscopy (T-200, JEOL, Japan). Proteoliposomes were applied to a carbon-coated grid and stained with 1% uranyl acetate as described by Haschmeyer and Myers [21].

Quantitation of residual detergent was done by modified spectrophotometric method [22]. The blue precipitates of Triton X-100 and ammonium cobalt thiocyanate complex were filtered by centrifugal filter devices (0.45 μm, Gelamn). The filtered precipitates were then solubilized by dichloromethane. A standard curve was made by plotting the absorbance at 630 nm and the amount of Triton X-100 under the same conditions of reconstitution such as the concentrations of salt, protein and phospholipid.

### 2.4. Transport assay

Transport was initiated by addition of 40 μM of [8-$^{14}$C]ATP (10 μl) to RER proteoliposomes (80 μl) along with 10 μl of other additives as specified in the individual experiments at 4 or 30°C. DIDS and ATR as additives were preincubated for 1 min under a dim light. The concentration of protein used for transport assay was routinely fixed as 0.1 mg/ml. Assays were terminated at selected time intervals by filtration through wet 0.22 μm cellulose filters (Millipore Corp., Bedford, MA, USA), followed by a wash with 5 ml of ice-cold reconstitution buffer containing 1 mM non-radiolabeled ATP. Filtration and rinsing were completed within 15 s. The filter was transferred from the ultrafiltration manifold into a scintillation vial, and 2 ml of scintillant was added. The radioactivity of the filter was measured by liquid scintillation spectrometry (Wallac Lsc model 1409) after the filter was completely dissolved in the scintillant.

Non-specific binding of a radiolabeled substance to RER proteoliposomes and filters was routinely assessed by mixing the proteoliposomes and the radiolabel at 4°C. This mixture was immediately subjected to the filtration procedure. Such blank values were < 0.1% of the total radioactivity applied to the filter and were subtracted from the test results.

### 2.5. Analytical methods

The protein was determined by the method of Peterson [23] with BSA as calibration standard. Phospholipid was measured by the method of Stewart [24].

## 3. Results

### 3.1. Optimization of reconstituted RER proteoliposomes

Initial experiments focused on developing the reconstitution conditions that represent the activity of ATP transport efficiently. In our previous paper, the minimum concentration of Triton X-100 to solubilize
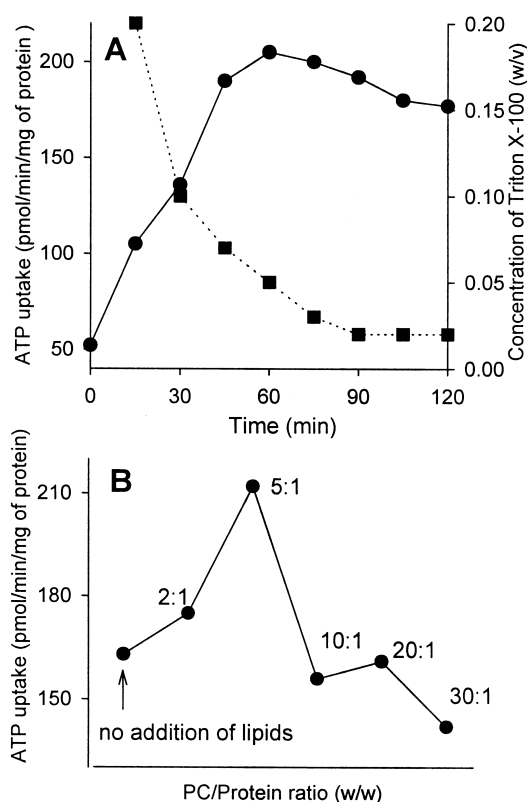
Fig. 1. Optimization of reconstitution parameters. RER vesicles were solubilized with the buffer containing 20 mM HEPES, pH 7.4, 3% (w/v) Triton X-100, 1 mM MgCl$_2$, 50 mM NaCl, 5% (w/v) glycerol and 1 mM PMSF for 30 min at 4°C. After separation of solubilized proteins from unextracted materials, the proteins were loaded on the Bio-beads SM-2 column and circulated. After a designated time, the amounts of residual detergent in the column eluates were measured and the eluates were mixed with the prepared liposomes. ATP transport activity was measured as described in Section 2. (A) ATP transport activity (●) and concentration of residual Triton X-100 (■) as a function of time of detergent-removal. (B) The activity was also checked for various phosphatidylcholine/protein ratios, where the time of removal of detergent was fixed at 60 min. Data represent the mean values of three preparations.

the ATP transporter effectively was determined by measuring the extent of the solubilized ATP transporter that had been photolabeled by radiolabeled ATP analogue [10]. The ratio of protein to detergent and the final concentration of Triton X-100 were adjusted to 1:2 (w/w) and 3% respectively. Thus, the time dependence of removal of detergent was first examined (Fig. 1A). Spectroscopic determination of residual Triton X-100 is less sensitive than the method using radiolabeled detergent [22], so the modified

procedure was developed. Rapid filtration of cobalt thiocyanate–Triton X-100 precipitates could be used to quantify the residual amount of detergent by the concentration of 0.02% (w/v) present in membranes during or after reconstitution of ATP transporter. ATP transport activity was the highest after detergent had been removed for about 1 h, that is, when the concentration of Triton X-100 was reduced to 0.05% (w/v). The loss or plateaus of ATP transport activity after 1 h of removal of detergent seemed to result from the denaturing effect when detergent was nearly removed. This time/activity value shows that a small but adequate amount of detergent is needed in the reconstitution of ATP transporter.

The PC/protein ratio is also thought to be dependent on the type of membrane protein used for reconstitution experiments. The result of the variation of this parameter is shown in Fig. 1B. Transport activity was tested with increasing concentration of liposomes and amount of fixed proteins. When the ratio was adjusted to 5:1 (PC:protein; w/w), the activity reached its maximum, going down drastically above a ratio of 10:1. This result suggests an unusual lipid dependence of ATP transporter considering that a ratio of PC/protein above 10:1 is currently used to reconstitute membrane proteins. The real PC/protein ratio of reconstituted proteoliposome was deter-
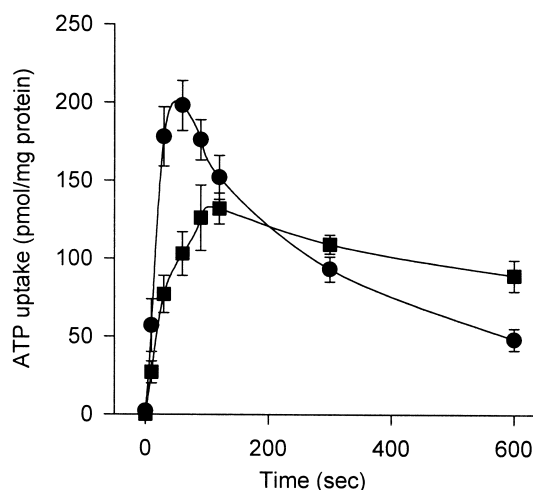


Fig. 2. Time and temperature dependence of ATP transporter. ATP transport activity was measured as a function of incubation time at 4°C (■) and 37°C (●). Transport reaction was started by addition of 40 μM of [8-$^{14}$C]ATP, which was preincubated at each temperature. Each data point represents the mean value for three experiments.

mined by measuring the amounts of the protein and lipid of proteoliposomes eluted in the void volume of a Sepharose 4B column. The real ratio of PC/protein showing the highest ATP transport activity was found to be 6:1. The slightly increased ratio is thought to be due to endogenous phospholipid and/or some loss of lumenal protein during reconstitution. The significant activity, which was shown when the liposomes were not added, seems to be due to the endogenous lipid, which includes various kinds of lipid components. Whether the specific lipid is needed for proper functioning of ATP transport needs to be examined. The size and homogeneity of proteoliposomes were also examined by electron microscopy. The reconstituted proteoliposomes were nearly homogeneous and had a shape of unilamellar vesicles with a mean diameter of about 75 nm (data not shown).

## 3.2. Characterization of ATP transport into RER proteoliposomes

Reconstituted ATP transporter made by optimizing some parameters of reconstitution was then characterized by its ATP transport activity. The activity
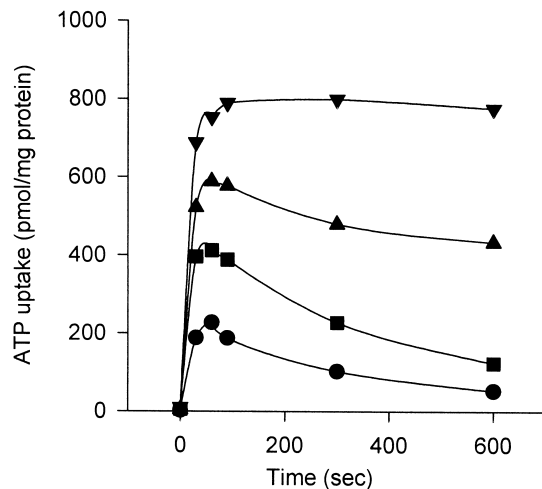


Fig. 3. The overshoot phase of ATP transport into RER proteoliposomes. ATP transport activity was measured as a function of incubation time at 37°C with various concentrations of external ATP. Transport reaction was started by addition of 10 μl of a designated concentration of [8-$^{14}$C]ATP, 50 μM (●), 100 μM (■), 200 μM (▲) and 500 μM (▼), which was preincubated at 37°C. Each data point represents the mean value for three experiments.
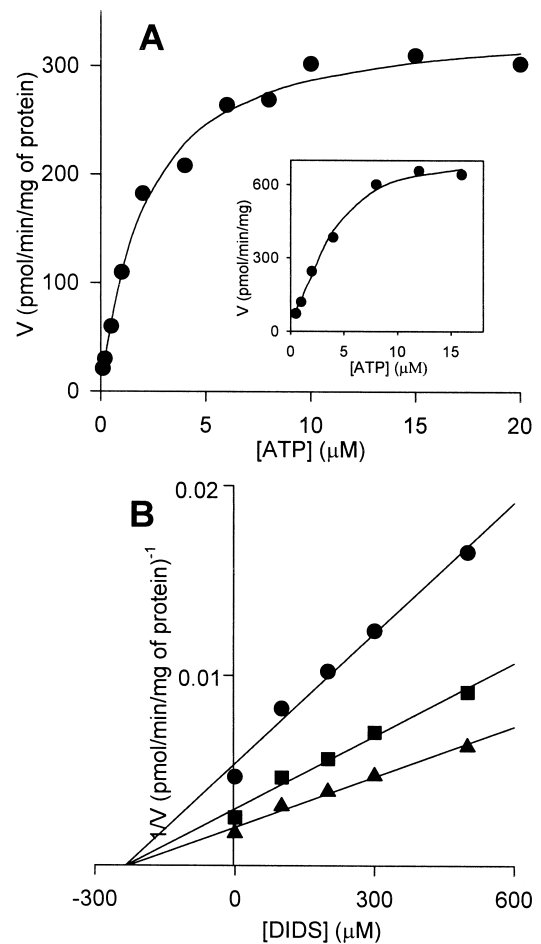


Fig. 4. Saturation and inhibitory behavior of ATP transporter. (A) Initial velocities of ATP transport activity of RER proteoliposomes were determined as a function of ATP concentration. Protein concentration was routinely fixed at 0.1 mg/ml. Incubation time and temperature were fixed at 1 min and 30°C respectively. (inset) ATP transport activity of intact RER. (B) Inhibition of ATP transport by DIDS. Each line in the Dixon plot represents data obtained at a constant [ATP], 4 μM (▲), 10 μM (■), and 20 μM (●). Data points represent the mean values for five experiments.

was measured by filtration method and corrected by subtracting the blank value, measured at the condition that radiolabeled ATP was incubated with RER proteoliposomes at 4°C for 10 s. Transport of ATP as a function of time is shown in Fig. 2. At 30°C, the transport was linear for 60 s, which represents an initial velocity. Therefore, the activity of ATP transporter in the RER proteoliposomes was determined by the measuring influx of [8-$^{14}$C]ATP at 30°C within the first 60 s. And the transport later displayed bi-

phasic kinetics, that is, an overshoot during which radioactivity inside the proteoliposomes transiently exceeded the amount of radiolabel present at later times, when transport equilibrium was approached. This overshoot phase was no longer observed when reaction temperature was adjusted at 4°C of temperature showing the relatively decreased initial velocity and the ATP concentration exceeded 500 μM (Fig. 3). Evidence that the overshoot phenomenon reflects *trans*-stimulation of ATP transport by counter substrates will be presented below.

The transport rates of ATP into proteoliposomes were plotted as a function of ATP concentration and the Lineweaver–Burk plot proved to be linear (data not shown). The $K_m$ value for ATP was 1.5 μM and the $V_{max}$ was 286 pmol/min/mg protein (Fig. 4A). These $K_m$ and $V_{max}$ values of RER proteoliposomes were lower than those of intact RER, $K_m$ of 6.5 μM and $V_{max}$ of 1 nmol/min/mg protein [10]. Although the $K_m$ and $V_{max}$ value for the reconstituted transporter were lower than those determined for the naïve transporter, the transport efficiencies were similar. This transport was also significantly inhibited by 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), known as an inhibitor of several anion transporters including ATP transporter of RER [7,8,10]. The Dixon plot shows that DIDS inhibited ATP transport in a non-competitive manner with a
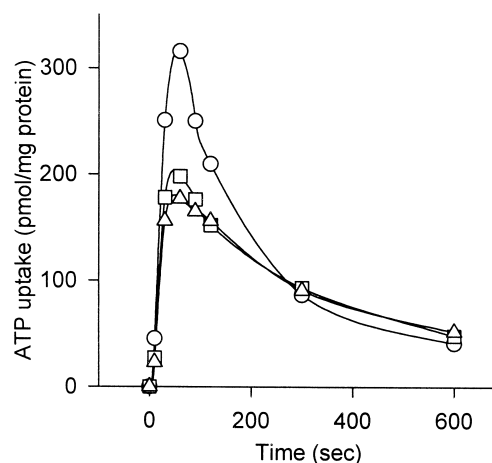


Fig. 5. *trans*-Stimulation of ATP transport by ADP. ADP-and AMP-preloaded proteoliposomes were constructed using reconstitution buffer containing 200 μM of ADP and ATP as described in Section 2. Time-course of ATP transport activity is displayed for ADP(○)-, AMP(△)- preloaded RER proteoliposomes and control (□). Data points represent the mean values for three experiments.

$K_i$ value of 240 μM (Fig. 4B). This value was compatible with the $K_i$ value of 230 μM in intact RER [10]. These results suggest that reconstituted RER proteoliposomes are fairly consistent with the ATP transport activity of intact RER.

### 3.3. cis- And trans-effects on ATP transport in RER proteoliposomes

The specificity of the ATP transporter was examined by the addition of structural analogues to the incubation medium (Table 1). AMP and ADP, which were added in 25-fold molar excess over the radiolabeled ATP, effectively inhibited ATP transporter activity. In the case where each nucleotide was added in 2.5-fold molar concentration, ATP transport was inhibited only by ADP. The other nucleotides did not inhibit ATP transport activity even in 25-fold molar excess. To determine more precisely whether this inhibition by ADP or AMP resulted from *cis*-inhibition, that is, counter substrate effect, ADP- and AMP-preloaded RER proteoliposomes were constructed to test their *trans*-stimulation (Fig. 5). The enhanced overshoot peak, compared with that of control proteoliposomes, was only shown in the time-dependence activity plot of ADP-preloaded proteoliposomes. In case of AMP-preloaded RER pro-

Table 1
*cis*-Inhibition of ATP transport by nucleotides

| Substrate | Concentration added (final, μM) | ATP transport activity (pmol/min/mg protein) |
|---|---|---|
| Control | | 202 ± 15 |
| ADP | 10 | 72 ± 18 |
| | 100 | 19 ± 3 |
| AMP | 10 | 174 ± 15 |
| | 100 | 89 ± 20 |
| GTP | 10 | 182 ± 11 |
| | 100 | 178 ± 12 |
| CTP | 10 | 205 ± 9 |
| | 100 | 181 ± 16 |
| UDP | 10 | 193 ± 15 |
| | 100 | 177 ± 12 |

Reconstituted RER proteoliposomes (80 μl, 0.1 mg of protein) were mixed with 10 μl of nucleotides as specified below for 10 min at 30°C. Control means the proteoliposomes where no nucleotides were preloaded. Transport was initiated by addition of 40 μM of [8-$^{14}$C]ATP (10 μl) to these reaction mixtures at 30°C. Data represented are ± S.D. for three experiments.

teoliposomes, the overshoot peak was slightly lower than that of control proteoliposomes. Thus, it was supposed that ATP transporter of RER was *cis*-inhibited and *trans*-stimulated by ADP only.

## 4. Discussion

The analysis and purification of membrane proteins such as transporters or carriers has been hampered by the fact that there are no universal methods to reconstitute them into artificial liposomes. Therefore, special precautions have to be taken to analyze the transport mechanism in the reconstituted system. In this paper, we described the functional reconstitution and characteristics of the ATP transporter in rat liver RER. In reconstituting the membrane proteins, especially by using the method of freeze–thaw-sonication, the proper time of removal of detergent and the lipid/protein ratio needed to be determined carefully. Although it is known that the removal of detergents by Bio-beads SM-2 is rapid and efficient in membrane transporters [25], trace amounts of detergent could have alternative effects such as denaturing of proteins or constructing proteoliposomes with decreased activity. The failure of the reconstitution of the ATP transporter in RER using the freeze–thaw-sonication method [16] might be because the subtle change of the reconstitution parameters could have a severe effect on the functional activity.

Reconstituted ATP transporters made by optimizing the parameters of reconstitution showed higher affinity for ATP and a lower $V_{max}$ value than intact RER. The former might be explained by the relative loss of activity of the other ATP-utilizing enzymes as suggested by Guillen and Hirschberg [16]. The latter is thought to be the result of the difference in the lumenal size and reversed orientation during reconstitution. The affinity for ATP of the ATP transporter in mammalian RER, deduced from intact or reconstituted RER, is higher than that in yeast RER [8]. This was also recognized through our preliminary experiments (data not shown) and showed the possibility that ATP transport into the RER of yeast and mammalian cells could be conducted by different proteins.

The reconstituted RER proteoliposomes showed the ATP transport activity and the inhibitory effect by DIDS, which were compatible with intact RER and suitable for the subsequent experiments of determining the ATP transport mechanism of RER. From the result of the effect of various external and some internal substrates on ATP transport activity, it was shown that ATP transport was both *cis*-inhibited and *trans*-stimulated by only ADP and it was supposed that the ATP transporter of mammalian RER has the ATP/ADP antiport mechanism. Although ATP transport activity was inhibited by external AMP which were added in 25-fold molar excess over the radiolabeled ATP, it was not *trans*-stimulated by internal AMP. On the other hand, ADP clearly *cis*-inhibited even in the 2.5-fold molar excess and *trans*-stimulated ATP transport activity of reconstituted RER proteoliposomes. Endogenous ADP seemed to exist still in RER proteoliposomes, although the concentration of ADP was lower than that of intact RER, which might be the reason that relatively low but apparent overshoot peak was also shown in control and AMP-preloaded proteoliposomes. Another possible explanation is the effect of rapidly degraded ADP when ATP is transported into the lumen. Transported ATP into the lumen of RER was used for various energy source of lumenal and/or membrane protein. Whether the ATP transporter itself has the ATP-hydrolysis activity or another ATP-utilizing enzyme exists in the RER membrane remains to be determined.

The ATP/ADP antiport mechanism of ATP transport into RER was same in yeast and mammalia. However, there should be more study to understand the relation between ATP transporter in RER and mitochondrial AAC because ATP transporter from RER had no significant inhibition by atractyloside or carboxyatractyloside, known as a specific inhibitor of mitochondrial AAC [10,26].

Under the optimized reconstitution parameters, purification of ATP transporter in RER and its peptide sequencing are in progress. These results will allow us to determine whether ATP transporter in mammalian RER is structurally related to yeast ATP transporter, Sac1p or mitochondrial AAC.

## Acknowledgements

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