Identification of the ATP Transporter of Rat Liver Rough Endoplasmic Reticulum via Photoaffinity Labeling and Partial Purification[†]

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ABSTRACT: In order to identify the ATP transporter in rat liver rough endoplasmic reticulum (RER), a photoreactive azido derivative of ATP, 3′-*O*-(*p*-azidobenzoyl)-ATP (AB-ATP), was synthesized by the reaction of ATP with *N*-hydroxysuccinimido 4-azidobenzoate (NHS-AB). The activity of the ATP transporter was determined by measuring the influx of [8-^{14}C]ATP. The ATP transport had an apparent K_m value of 6.5 μM and a V_{max} of 1 nmol min^{-1} (mg of protein)$^{-1}$. The transport of ATP was specifically inhibited by AB-ATP and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). Under a dim light, AB-ATP was a competitive inhibitor of the ATP transport with a K_i value of 0.19 μM, which indicates that AB-ATP has a high affinity for the ATP transporter, so it can be utilized as a photoaffinity probe for the identification of the ATP transporter in rat liver RER. An SDS−PAGE analysis of RER vesicles photolabeled with [γ-^{32}P]AB-ATP indicates the presence of a 56-kDa protein. The 56-kDa protein was completely protected from photoaffinity labeling by 10 μM ATP but not by 30 μM GTP. The specific labeling of the 56-kDa protein was sensitive to the anion transport inhibitor DIDS. In order to confirm whether the apparent uptake of ATP was due to the 56-kDa protein, the ATP transporter was partially purified through two successive ion-exchange chromatography steps (DEAE and Mono-S). The fraction showing the high activity of the ATP transporter also contained the 56-kDa protein photolabeled with [γ-^{32}P]AB-ATP. On the basis of the photoaffinity labeling and reconstitution experiment, we conclude that the 56-kDa protein represents the ATP transporter in rat liver RER.

Rough endoplasmic reticulum (RER)[1] membranes are highly sensitive permeability barriers because they contain specific molecular pumps and transporters. These transport systems regulate the molecular and ionic composition of RER. ATP, an effective energy source in various biological processes, is synthesized within the mitochondria and exchanged with the cytosolic ADP by the action of the mitochondrial ADP/ATP carrier (AAC) (Klingenberg, 1976). Since it has been known that ATP is used for efficient assembling and folding of proteins in the lumen of RER (Klappa *et al*., 1991; Flynn *et al*., 1989) and for transport of proteins from ER to Golgi complex (Dorner *et al*., 1990), a ATP transport system should exist across RER membranes.

Recently, Clairmont *et al*. (1992) reported that rat liver and canine pancreas RER-derived vesicles can transport ATP into their lumen in a protein-mediated saturable manner. In an analogous study, Mayinger and Meyer (1993) showed that yeast ER possesses an enzyme-mediated ATP transport system, which is essential for the translocation of preproteins across the RER membrane.

Although it has been known that ATP transport into RER vesicles occurs in a protein-mediated manner, none of the proteins responsible for ATP transport in the RER have been identified. In order to identify the ATP transporter isolated from RER vesicles, we used the photoaffinity labeling technique, a powerful tool to enable highly specific labeling of membrane proteins (Shanahan *et al*., 1985; Weber & Eichholz, 1985), and *in vitro* reconstitution into artificial membranes as a functional assay (Levy *et al*., 1992). In addition, to obtain a clear understanding of the ATP transporter, we have partially purified the ATP transporter, sustaining its activity subsequent to its incorporation into liposomes generated from microsomal lipids.

In this report, the specific photoaffinity labeling and *in vitro* reconstituted assay system can be utilized as tools for the identification of the ATP transporter of rat liver RER.

MATERIALS AND METHODS

Materials. Female Wistar rats were obtained from Seoul National University Breeding Laboratories. The following radioactive nucleotides were purchased from Amersham Corp.: [γ-^{32}P]ATP, 3000 Ci/mmol, and [8-^{14}C]ATP, 51 mCi/mmol. Chemicals were obtained from the following sources: ATP, GTP, NHS-AB, DIDS, ATR, HEPES, Dowex (1X8, chloride form, 100−200 mesh), DEAE-cellulose, and prestained markers composed of pyruvate kinase (58 kDa) and triosephosphate isomerase (26.6 kDa) were from Sigma. Molecular weight markers for SDS−PAGE were from Bio-Rad. Silica TLC aluminum plate (60 F 254, 5554) was purchased from Merck. Microcon (0.22 μm) was purchased from Amicon.

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[1] Abbreviations: RER, rough endoplasmic reticulum; AB-ATP, 3′-*O*-(*p*-azidobenzoyl)adenosine 5′-triphosphate; AAC, ADP/ATP carrier; BiP, immunoglobulin heavy-chain binding protein; NHS-AB, *N*-hydroxysuccinimido 4-azidobenzoate; GTP, guanosine 5′-triphosphate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; TEAB, triethylammonium bicarbonate; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; DEAE, diethylaminoethyl; FPLC, fast protein liquid chromatography.

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Table 1: Enzyme Marker Analysis for Isolated Subcellular Fractions[a]

	cytochrome *c* oxidase		α-mannosidase II		alkaline phosphodiesterase I		glucose-6-phosphatase	
fraction	SA	RSA	SA	RSA	SA	RSA	SA	RSA
homogenate	0.66	1.00	0.11	1.00	0.43	1.00	0.72	1.00
mitochondria	1.32	2.00	0.17	1.54	0.59	1.37	0.17	0.24
Golgi	0.66	1.00	0.62	5.63	1.81	4.21	0.32	0.44
microsome	0.55	0.83	0.13	1.18	1.35	3.14	3.50	4.86
RER	0.10	0.15	0.06	0.54	0.05	0.12	3.60	5.00

[a] Subcellular fractions (mitochondria, Golgi apparatus, crude microsome, RER) were prepared as described under Materials and Methods. The enzymes indicated were assayed in each fraction and the data were given as the specific activity (SA, units per milligram of protein) and as the relative specific activity (RSA, $SA_{fraction}/SA_{homogenate}$). One unit of activity corresponds to 1 μmol of substrate changed/min.

Subcellular Fractionation. Liver subcellular organelles (mitochondria, Golgi, microsome, rough endoplasic reticulum) were fractionated from the livers of female Wistar rats, which had been starved for 16−20 h prior to sacrifice by anesthesia, according to Fleischer and Kervina (1974). RER vesicles were resuspended in buffer A at a concentration of 5 mg of protein/mL and stored at −70 °C. The characterization of the subcellular fractions was accomplished by marker enzyme assay (Table 1). Cytochrome *c* oxidase activity was determined by the procedure of Madden and Atorrie (1987), and *a*-mannosidase II was assayed by the method of Tulsiani *et al*. (1982). The alkaline phosphodiesterase I activity was monitored by the method of Beaufay (1974). The determination of free inorganic phosphate released by the activity of glucose-6-phosphatase was performed by the method of Ames (1966). RER vesicles were at least 92% intact, based on latency of glucose-6-phosphatase toward mannose 6-phosphate (Arion *et al*., 1975). The concentration of protein was determined by the method of Peterson (1977).

Synthesis of 3′-O-(p-Azidobenzoyl)adenosine Triphosphate. NHS-AB (45.5 mg, 0.175 mmol) was dissolved in 0.2 mL of dimethylformamide, followed by the addition of 0.2 mL of ATP (20.1 mg, 0.035 mmol) in 0.24 M TEAB buffer, pH 7.6. The reaction was allowed to proceed for 4−8 h at room temperature with mild stirring. The cloudy mixture was then separated on preparative silica TLC (Merck) plates (20 cm × 20 cm) with the solvent mixture 1-butanol/H_2O/CH_3COOH (5:3:2 v/v/v). The AB-ATP band (R_f = 0.35), separated clearly from ATP (R_f = 0.1), NHS-AB (R_f = 0.9), azidobenzoic acid (R_f = 0.95), and *N*-hydroxysuccinimide (R_f = 0.72), was scraped off and extracted with water. The extract was filtered by Microcon (0.22 μm) and the filtrate was lyophilized and assayed. The components of the reaction mixture and the purity of separated AB-ATP were confirmed by analytical HPLC (Vydac 303 NT 50 × 4.6). Solvent systems were solvent A (45 mM KH_2PO_4, pH 4.6) and solvent B (500 mM KH_2PO_4, pH 2.7). Elution was carried out at a flow rate of 2.5 mL/min. [γ-^{32}P]AB-ATP was prepared in an identical manner, utilizing [γ-^{32}P]ATP (100 μCi). The yield, estimated by liquid scintillation spectrometry, was about 50% with respect to the added [γ-^{32}P]ATP.

Transport Assay. The transport of ATP into RER vesicles was assayed by an ion-exchanger (Dowex) method which is a modified method originally described by Mayinger and Meyer (1993). RER vesicles (100 μg of protein) were preincubated in 0.25 M sucrose, 10 mM HEPES, pH 7.4, and 1 mM $MgCl_2$ at 30 °C for 1 min with or without the competitor, such as DIDS and AB-ATP, under a dim light and then incubated for an additional 1 min with different concentrations (from 1 to 10 μM) of [8-^{14}C]ATP at 30 °C. The ATP transport was stopped by rapid mixing of RER vesicles with 70 mg of Dowex (1X8, chloride form, 100−200 mesh), which was preequilibrated with 0.17 M sucrose and 3 mg/mL BSA, in an 1.5-mL eppendorf tube for 1 min on ice. The suspensions were transferred to a blue tip (1 mL) sealed with glass fiber tape and then RER vesicles were immediately eluted by Gilson pipetman. Under this method more than 90% of the proteins were recovered in the eluate, whose radioactivity was quantified by liquid scintillation spectrometry. The process involved in taking and collecting RER vesicles was completed within a few seconds and the latency of mannose-6-phosphatase of the recovered RER vesicles was maintained.

Photoaffinity Labeling and SDS−PAGE. The photoaffinity labeling of [γ-^{32}P]AB-ATP was accomplished as follows: RER vesicles (100 μg of protein) were preincubated in the dark with or without known competitors (DIDS, ATP, GTP, and atractyloside) on ice for 10 min. The photoaffinity labeling was initiated by addition of 30 nM [γ-^{32}P]AB-ATP. After a 2-min incubation at room temperature, the samples were photolyzed with UV irradiation (254 nm, 25 μW/cm^2) for 1 min at a distance of 5 cm. The reaction was quenched by addition of 0.25 volume of 4× SDS−PAGE sample buffer consisting of 250 mM Tris-HCl, pH 6.8, 8% SDS, 20% β-mercaptoethanol, 40% glycerol, and bromophenol blue (tracking dye). The samples were subjected to SDS−PAGE using a 10% resolving gel according to the method of Laemmli (1970). After electrophoresis, the gel was stained with Coomassie Brilliant Blue, destained, dried, and subjected to autoradiography. Molecular weights were estimated by using the following standards: phosphorylase *b* (97 000), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), and trypsin inhibitor (21 500).

Preparation of Liposomes and Reconstitution. Endogenous lipids were extracted from fresh microsomal pellets according to the method of Folch *et al*., (1957) and suspended in buffer B composed of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, and 1 mM DTT. Phospholipid concentration was determined using the method of Stewart (1980). Liposomes were prepared by sonication in a bath-type sonicator until an almost clear suspension was obtained. RER proteins were solubilized by the addition of an equal volume of buffer C (100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 20% glycerol, and 4% Triton X-100), where the ratio of protein to detergent was adjusted to 1:2 (w/w). After incubation on ice for 30 min, the mixture was centrifuged for 1 h at 105000*g*. To remove Triton X-100, SM-2 Bio-Beads, which were extensively washed before use as described by Holloway (1973), were added to the solubilized proteins at a concentration of 100 mg of wet beads/mL. The

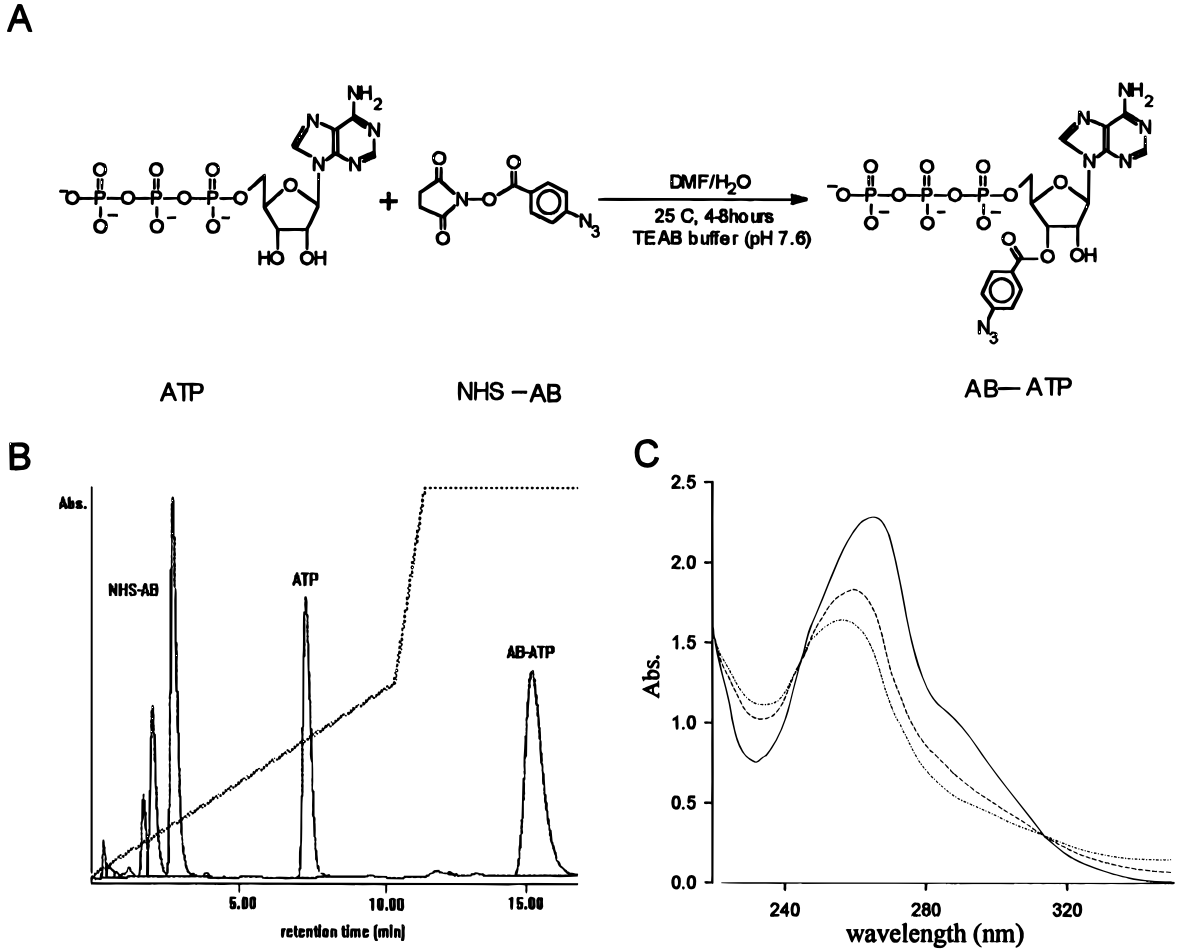


FIGURE 1: (A) Schematic representation of the synthesis of AB-ATP. (B) HPLC (Vydac 303NT 50 × 4.6) analysis of the reaction mixture. Solvent systems were solvent A (45 mM KH_2PO_4, pH 4.6) and solvent B (500 mM KH_2PO_4, pH 2.7). Elution was carried out at a flow rate of 2.5 mL/min. (C) Effect of irradiation on the absorption spectrum of AB-ATP. AB-ATP (21.8 μM) was irradiated by UV light (254 nm, 24 μW/cm^2) in H_2O at 24 °C and the absorption spectra were recorded after varying periods of time. (—) Unirradiated; (- - -) 5-min irradiation; (-·-) 10-min irradiation.

mixtures were gently stirred in a cold room (4 °C) for 3 h. It is known that less than 1% of the initial Triton X-100 was detected after 3 h of incubation (Rigaud *et al.*, 1988). The detergent-removed proteins were added to the preformed liposomes, where the final protein to liposome ratio was adjusted to 1:5 (w/w). The mixture was then quickly frozen in liquid nitrogen and allowed to thaw slowly at room temperature. The freeze−thaw cycle was repeated four times to form large and homogenous proteoliposomes, which were then sonicated for 10 s. These resultant proteoliposomes were directly used for transport assay.

Partial Purification of the ATP Transporter. The solubilized RER proteins (10 mg) were applied to a DEAE-cellulose column (1.4 × 22 cm) equilibrated with buffer D (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.5% Triton X-100) at a flow rate of 30 mL/h. The column was washed with 1 column volume of buffer D, then with buffer D containing 0.2 M NaCl, and finally with buffer D containing 0.5 M NaCl. The protein concentration of each fraction was determined by the method of Peterson (1977). Each fraction of DEAE chromatography was photolabeled with [γ-^{32}P]AB-ATP and reconstituted with liposomes to assay the ATP transport activity. The active fractions were pooled and exchanged with buffer E (20 mM MOPS, pH 7.0, 1 mM EDTA, 10 mM NaCl, 10% glycerol, and 0.2% Triton X-100) and then concentrated with Amicon ultrafiltration (10 000 MW cutoff). The concentrated sample (1 mg) was applied to a Mono-S HR 5/5 (Pharmacia) prepacked cation-exchange column (50 × 5-mm i.d.) which was installed in a fast protein liquid chromatography (FPLC) system and then eluted with a linear NaCl gradient (10−500 mM) in buffer E at a flow rate of 30 mL/h. Fractions (0.5 mL) of the eluate were collected, assayed for ATP transport activity by the reconstitution procedure, and photolabeled with [γ-^{32}P]AB-ATP.

RESULTS

Synthesis of AB-ATP and Its Photolability. The synthetic scheme of AB-ATP and its structure is shown in Figure 1A. The synthetic yield is about 50%. The reaction mixture had five discrete UV-absorbing spots on silica TLC. HPLC analysis showed the same number of peaks, suggesting the purity of each band (Figure 1B). AB-ATP was comprised of ATP and a photoreactive azidoaryl group coupled via an ester bond to the 3′-hydroxyl group of the ribose ring of ATP. Purified AB-ATP was completely hydrolyzed to azidobenzoic acid (R_f 0.95) and ATP (R_f 0.1) in the basic condition of 1 M NH_4HCO_3, which showed the existence of a base-labile ester linkage. The UV spectrum (Figure 1C) showed a λ_{max} of 266 nm in H_2O and the molecule had a molar extinction coefficient of 9.2 × 10^4, which was determined by total phosphate analysis of AB-ATP. Irradiation of AB-ATP with UV light (254 nm) caused photodecomposition and λ_{max} was shifted from 266 to 258 nm.

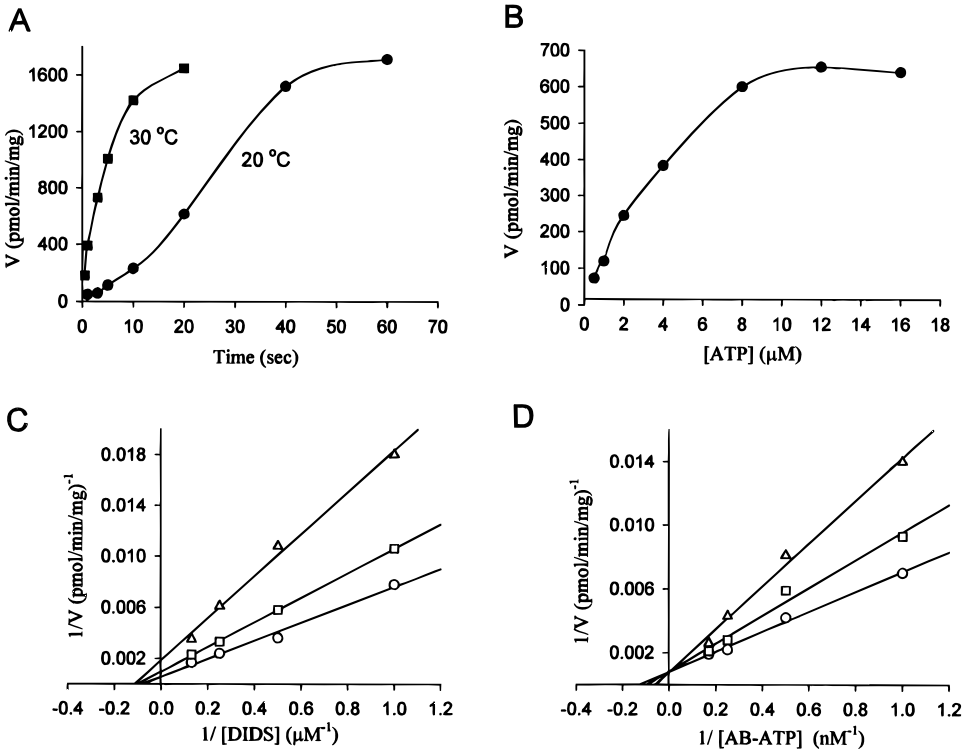


FIGURE 2: ATP transport into RER vesicles. RER vesicles (100 μg of protein) were preincubated in 0.25 M sucrose, 10 mM HEPES, pH 7.4, and 1 mM $MgCl_2$ at 30 °C for 1 min with or without the competitor, such as AB-ATP and DIDS, under a dim light and then incubated an additional 1 min with different concentrations (from 1 to 20 μM) of [8-^{14}C]ATP at 30 °C. ATP transport was measured using the ion-exchanger (Dowex) method described under Materials and Methods and corrected by substracting the amount of ATP associated with deoxycholate-permeabilized RER vesicles. Data were from four independent measurements. (A) Time course of ATP uptake in RER vesicles. RER vesicles (100 μg of protein) were incubated with 4 μM ATP at either 30 or 20 °C. (B) Rate of ATP transport into rat liver RER vesicles versus ATP concentration. The Lineweaver−Burk plot (not shown) gave a K_m of 6.5 μM and a V_{max} of 1 nmol min^{-1} (mg of protein)$^{-1}$. (C) Noncompetitive inhibition of the ATP transport by DIDS in the dark. Control (○); plus 100 μM (□); plus 200 μM DIDS (△). (D) Competitive inhibition of the ATP transport by AB-ATP in the dark. Control (○); plus 100 nM AB-ATP (□); plus 200 nM AB-ATP (△).

The isosbestic points at 244 and 314 nm, which indicate a single reactant going to a single product, reconfirmed the purity of AB-ATP.

Transport Studies for the ATP Transporter. The ATP transport into RER vesicles was measured using an ion exchanger (Dowex). The activity of the ATP transporter was corrected by substracting the amount of ATP associated with 0.25% deoxycholate-permeabilized RER vesicles, so the assay can exclude the amount of ATP induced by ATP binding and hydrolysis in the lumen. The uptake of ATP as a function of time and temperature is shown in Figure 2A. At 30 °C, the transport was seem to be linear for 60 s, which represents an initial velocity. Therefore, the activity of the ATP transporter was determined by measuring the influx of [8-^{14}C]ATP in 0.25 M sucrose, 10 mM HEPES, pH 7.4, and 1 mM $MgCl_2$ at 30 °C within the first 60 s. The transport rates were plotted as a function of ATP concentration (Figure 2B) and the Lineweaver−Burk plot (Lineweaver & Burk, 1934) proved to be linear (data not shown). The K_m value for ATP was 6.5 μM and the V_{max} was 1 nmol min^{-1} (mg of protein)$^{-1}$. Our K_m value was compatible with those of rat RER (4 μM; Clairmont *et al.*, 1992) and yeast ER (10 μM; Mayinger & Meyer, 1993). The effect of DIDS, a potent and nonpermeating inhibitor of anion transport (Cabantchik *et al.*, 1978; Ehrenspeck & Brodsky, 1976), on the ATP transport is shown in Figure 2C. The Lineweaver−Burk plot suggest that DIDS is a noncompetitive inhibitor with a K_i value of 230 μM. However, atractyloside, a specific inhibitor of the mitochondrial AAC (Vignais, 1976), had no effect on ATP transport, which rules out any chance of mitochondrial contamination. Even though the structure of AB-ATP is very similar to that of ATP, it was essential to test the probe's ability to inhibit the ATP transport in the absence of UV light. Figure 2D shows a Lineweaver−Burk plot of the inhibition effect of AB-ATP at various concentrations of ATP. It is apparent that AB-ATP specifically inhibits the ATP uptake with a K_i value of 0.19 μM. This result demonstrates that AB-ATP can be used to photolabel the ATP recognition site with high efficiency and that use of [γ-^{32}P]AB-ATP can lead to the identification of the ATP transporter.

Photoaffinity Labeling of RER Vesicles with [γ-^{32}P]AB-ATP. An SDS−PAGE analysis for photoaffinity labeling of each subcellular fraction is shown in Figure 3. In mitochondria, three proteins were photolabeled (Figure 3, lane 1), and a 35-kDa protein seemed to be the mitochondrial AAC in consideration of its molecular mass. In the Golgi apparatus, a 62-kDa protein was weakly photolabeled (Figure 3, lane 2). In microsomes (Figure 3, lane 3), several proteins were photolabeled. These might result from contaminating cytosol, lysosomes, and plasma membranes in comparison with the labeling pattern in highly purified RER vesicles. Surprisingly, photolabeled RER vesicles showed only one ^{32}P-labeled band with an apparent molecular size of 56 kDa (Figure 3, lane 4). Therefore, the following experiments were carried out with highly purified RER vesicles to exclude contaminating proteins from other subcellular organelles.

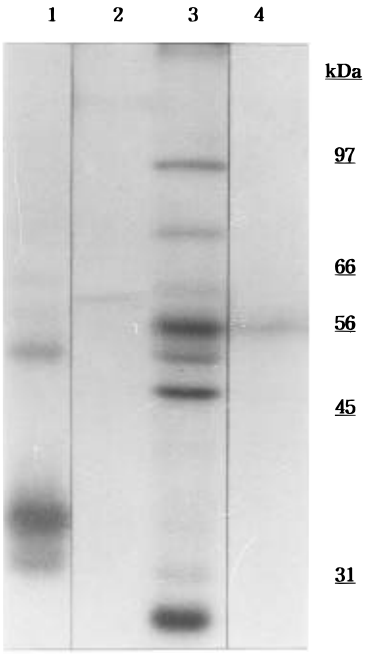


FIGURE 3: Photoaffinity labeling of subcellular organelles with [γ-^{32}P]AB-ATP. Each subcellular organelle (100 μg of protein) was incubated with 30 nM [γ-^{32}P]AB-ATP for 2 min at room temperature and then photolyzed with UV irradiation (254 nm, 25 μW/cm^2) for 1 min at a distance of 5 cm. Lane 1, mitochondria; lane 2, Golgi apparatus; lane 3, microsomes; lane 4, RER vesicles.

Competition Experiments against the Photoaffinity Labeling of the RER Vesicles with [γ-^{32}P]AB-ATP. The specific photoaffinity labeling of the 56-kDa protein was significantly protected with nonradioactive ATP (Figure 4A). ATP afforded almost complete protection against the photoaffinity labeling at 10 μM, but GTP did not affect the specific labeling (Figure 4B). DIDS specifically reduced the radioactive intensity of the 56-kDa protein band (Figure 4C), and atractyloside did not affect the specific labeling (data not shown).

Solubilization and Reconstitution of the ATP Transporter. In order to optimize the solubilization conditions of the protein, we examined the solubilization efficiency under various conditions using the photolabeled RER vesicles. The selective extraction of the ATP transporter from RER vesicles was performed in a buffer containing 50 mM NaCl and 2% Triton X-100 to adjust the protein:detergent ratio to 1:2. The solubilized proteins were then reconstituted with liposome using the freeze−thaw sonication method (Levy *et al*., 1992). The ATP transport into RER proteoliposomes was closely similar to that into RER vesicles. The ATP transport was inhibited by AB-ATP and DIDS but not by atractyloside (Figure 5). Liposomes alone did not show transport activity. These results demonstrate that the reconstituted system can be used as a powerful tool for the identification of the ATP transporter.

Partial Purification of the ATP Transporter. We tried to purify the ATP transporter in order to prove that the activity of the ATP transporter in the reconstituted proteoliposomes was from the 56-kDa protein. The ATP transporter was extracted with Triton X-100 from rat liver RER membranes and the solubilized proteins was applied to a DEAE-cellulose column followed by elution of the column with an NaCl step gradient (0.05, 0.2, and 0.5 M NaCl) in buffer D. Then, collected proteins were reconstituted into liposomes to assay

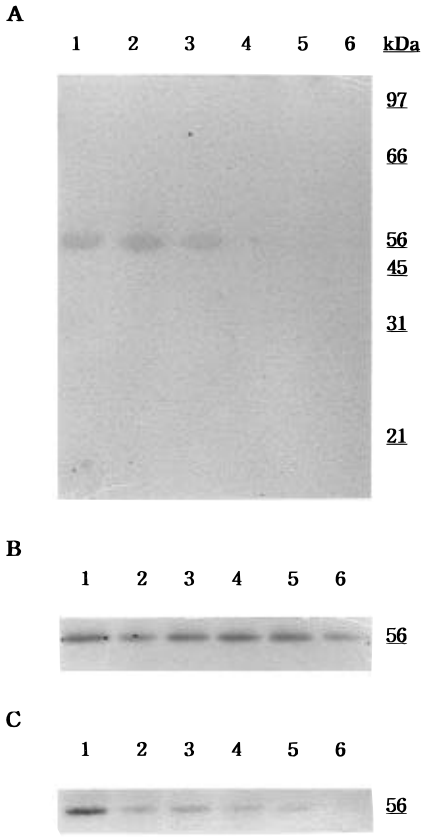


FIGURE 4: Specificity of the photoaffinity labeling of RER vesicles with [γ-^{32}P]AB-ATP. (A) Nonradioactive ATP prevents the 56-kDa protein from being photoaffinity labeled with [γ-^{32}P]AB-ATP. Lane 1, 1 μM ATP; lane 2, μM ATP; lane 3, 5 μM ATP; lane 4, 10 μM ATP; lane 5, 20 μM ATP; lane 6, 30 μM ATP. (B) Specific photoaffinity labeling of the 56-kDa protein is independent of nonradioactive GTP. Lane 1, 0 μM GTP; lane 2, 2 μM GTP; lane 3, 5 μM GTP; lane 4, 10 μM GTP; lane 5, 20 μM GTP; lane 6, 30 μM GTP. (C) DIDS specifically inhibits the photoaffinity labeling of the 56-kDa protein. Lane 1, 0 μM DIDS; lane 2, 100 μM DIDS; lane 3, 200 μM DIDS; lane 4, 300 μM DIDS; lane 5, 500 μM DIDS; lane 6, 700 μM DIDS.

the activity of the ATP transporter and photolabeled with [γ-^{32}P]AB-ATP. Only pool 1, eluted with 50 mM NaCl, had high activity of the ATP transporter and contained the 56-kDa protein photolabeled with [γ-^{32}P]AB-ATP (Figure 6). The ATP transport activity was increased 4.3-fold during this step (Table 2). The active fractions (pool 1) from the DEAE-cellulose chromatography were loaded onto a Mono-S FPLC column (Figure 7). The ATP transport activity was observed in fractions which eluted in a 0.1−0.15 M NaCl gradient range from the Mono-S FPLC column. These procedures resulted in at least 33-fold purification. The recoveries and specific activities during the partial purification are shown in Table 2. Enhanced purity after this step was verified by SDS−PAGE analysis and autoradiograms. The photoaffinity labeling of the active fraction from the two successive columns showed the photolabeled 56-kDa protein photolabeled with [γ-^{32}P]AB-ATP (Figure 8). During the partial purification, a new 32-kDa protein was photolabeled with [γ-^{32}P]AB-ATP as strongly as the 56-kDa protein in DEAE and Mono-S active fractions, but not in the solubilized RER proteins. Provisionally, the 32-kDa protein may be the digested fragment of the 56-kDa protein during purification.

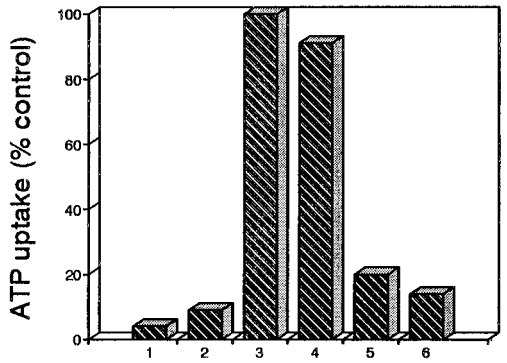


FIGURE 5: ATP transport into RER proteoliposomes. RER proteoliposomes were prepared by the freeze−thaw sonication method described under Materials and Methods. RER proteoliposomes (100 μg of protein) were preincubated in 0.25 M sucrose, 10 mM HEPES, pH 7.4, and 1 mM $MgCl_2$ on ice for 10 min with or without additives such as deoxycholate, ATR, DIDS, and AB-ATP under a dim light and then incubated an additional 5 min with 4 μM of [8-^{14}C]ATP at room temperature. The transport assay was performed according to Materials and Methods. Inhibition (percent) of ATP transport into RER proteoliposomes by ATR, DIDS, and AB-ATP is described. Bar 1, liposomes (without proteins); bar 2, 0.25% deoxycholate-treated proteoliposomes; bar 3, RER proteoliposomes; bar 4, 100 μM ATR-treated proteoliposomes; bar 5, 500 μM DIDS-treated proteoliposomes; bar 6, 50 μM AB-ATP-treated proteoliposomes.

DISCUSSION

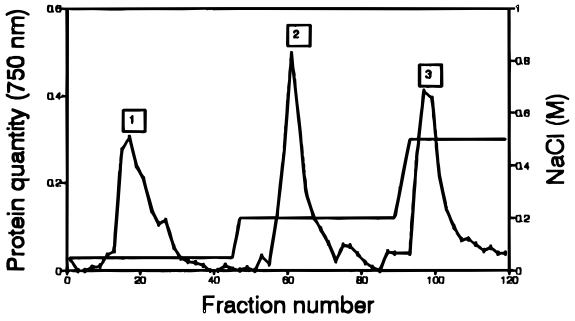
It is interesting to determine how ATP can be transported from the cytosol to the lumen of RER, so we utilized the photoaffinity labeling technique to identify the ATP transporter of rat liver RER. In practice, it is desirable to introduce only minor changes in the structure of ATP in designing the ATP analogue. Therefore, the analogue will bind to the ATP recognition site almost as ATP does, and the photoaffinity labeling will have high specificity.

AB-ATP can be found in the writings of Guillory and Jeng (1977). They described great deal of the chemistry about the esterification of the ribose hydroxyl group of ATP and showed much use of azidoaryl-β-alanyl-ATP in biological systems. Contrary to the extensive use of azidoaryl-β-alanyl-ATP, the utilization of AB-ATP, used in our experiments, was rarely reported. Thus the results reported here also indicate that AB-ATP will be very useful for studying various aspects of ATP-utilizing enzymes.

AB-ATP exhibits more desirable photochemical properties than other azido derivatives of ATP which have been currently used in the photoaffinity labeling of the adenosine nucleotide carrier. First, the adenine ring of AB-ATP is left unmodified, so it can specifically bind to the ATP transporter with almost the same affinity as ATP. Second, the aryl group of AB-ATP lends a greater chemical stability to the analogue than other azides and also imparts a significant hydrophobic character to the molecule. This hydrophobic photoreactive reagent can be used to label the exposed surfaces of the integral proteins associated with the lipid bilayer (Weber & Eichholz, 1985; Shanahan *et al*., 1985).

The usefulness of AB-ATP as a potential photoaffinity probe for the active site of the ATP transporter is verified by the inhibitory property of AB-ATP for ATP transport into RER vesicles. Figure 2C,D shows that the inhibition property of AB-ATP (K_i value of 0.19 μM) for ATP transport is almost 1000-fold stronger than that of DIDS (K_i value of 230 μM), indicating that AB-ATP is a more specific inhibitor

A



B

	Pool 1	Pool 2	Pool 3
Activity (pmol/mg/5min)	1.41	0.18	0.11

C

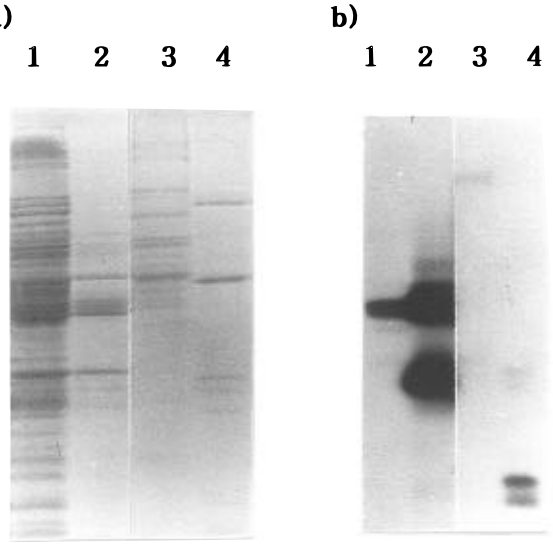


FIGURE 6: Chromatography on DEAE-cellulose. RER vesicles were solubilized with Triton X-100 and then the solubilized proteins (10 mg) were applied to a column (1.4 × 22 cm) of DE 52. The column was subsequently eluted with different NaCl concentrations (50, 200, and 500 mM) at a flow rate of 30 mL/h and 1.5-mL fractions were collected. Each fraction was assayed for protein quantity by using the Peterson method. Absorbance at 750 nm was monitored (■). The labels 1, 2, and 3 refer to pools used in successive transport assay and photoaffinity labeling with [γ-^{32}P]AB-ATP. (A) Profile of DEAE chromatography. (B) ATP transport activity of each pool. (C) Photoaffinity labeling of each pool with [γ-^{32}P]AB-ATP. (a) The protein pools from DEAE-cellulose were analyzed on an SDS−10% polyacrylamide gel in the following amounts and stained with Coomassie Blue R-250: lane 1, RER, 100 μg; lane 2, pool 1, 50 μg; lane 3, pool 2, 50 μg; lane 4, pool 3, 50 μg. (b) Autoradiogram of the SDS−polyacrylamide gel.

than DIDS. The results demonstrate that AB-ATP photolabels the ATP transporter with good efficiency and that use of [γ-^{32}P]AB-ATP can lead to the identification of the ATP transporter in rat liver RER.

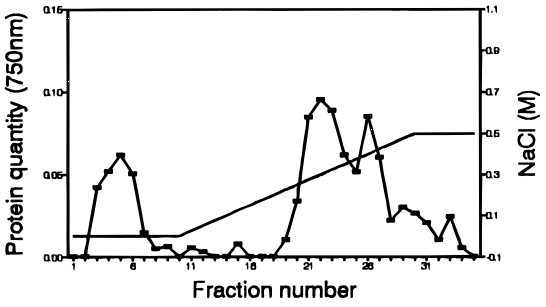
In various competition experiments using ATP, GTP, DIDS, and atractyloside, it is shown that the photoaffinity labeling of the 56-kDa protein is specifically inhibited by nonradioactive ATP and DIDS but not by nonradioactive

Table 2: Partial Purification of the ATP Transporter in Rat Liver RER[a]

fraction	ATP transporter protein (mg)	specific activity [pmol mg^{-1} (5 min)$^{-1}$]	purification (x-fold)
solubilized proteins	10.0	0.32	1.00
DEAE (0.05 M NaCl)	1.0	1.41	4.41
Mono-S (0.15 M NaCl)	0.3	10.39	32.47

[a] RER vesicles (30 mg of protein) were solubilized with 2% Triton X-100 and the solubilized proteins (10 mg of protein) were applied to the DEAE-cellulose column followed by the Mono-S FPLC. Then, the protein in a highly purified state was reconstituted with endogenous microsomal liposomes. The RER proteoliposomes were incubated for 5 min at room temperature with 4 μM [8-^{14}C]ATP. The activity of the ATP transporter was assayed as described under Materials and Methods.

A



B

Fraction #	5	21	27
Activity (pmol/mg/5min)	1.44	10.39	0.56

FIGURE 7: Chromatography on Mono-S (FPLC). The active fractions (pool 1) from the DEAE-cellulose column (1 mg) were loaded onto a Mono-S HR 5/5 prepacked cation-exchange column. The flow rate was 30 mL/h. The column was washed with 5 column volumes of buffer E composed of 20 mM MOPS, pH 7.0, 1 mM EDTA, 10 mM NaCl, 10% glycerol, and 0.2% Triton X-100 and then eluted with a linear NaCl gradient (from 10 to 500 mM) in buffer E. Fractions (0.5-mL) were collected. (A) Profile of Mono-S (FPLC) chromatography. (B) ATP transport activity of each fraction. The fractions were reconstituted with endogenous microsomal liposomes to assay the ATP transport activity.

GTP and atractyloside. These results are consistent with the inhibitory property of nonradioactive ATP and DIDS for ATP transport, strongly suggesting that the 56-kDa protein can be the ATP transporter in rat liver RER vesicles.

ATP transport into RER vesicles (K_m value of 6.5 μM) is compatible with ATP transport into yeast ER (K_m value of 10 μM). The effect by atractyloside on ATP transport across RER membranes is different than that reported for the inhibition of ADP/ATP transport into mitochondria. It is suggested that the mitochondrial AAC and the ATP transporter of RER vesicles have different structural features.

Transporter of ATP into RER proteoliposomes is closely similar to transport into RER vesicles with regard to inhibitor characteristics (Figure 5). This reconstituted system can be used to identify the ATP transporter.

Partial purification of the ATP transporter enables us to confirm that the uptake of ATP is from the 56-kDa protein. During two successive ion-exchange chromatography steps

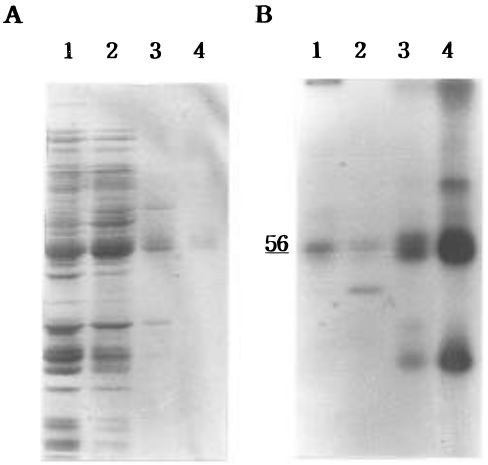


FIGURE 8: Photoaffinity labeling of the partially purified ATP transporter with [γ-^{32}P]AB-ATP. (A) Coomassie blue-stained SDS−polyacrylamide gel. (B) Autoradiogram of the SDS−polyacrylamide gel. Lane 1, RER, 100 μg; lane 2, soluble RER proteins, 100 μg; lane 3, DEAE pool, 20 μg; lane 4, Mono-S pool, 10 μg.

(DEAE and Mono-S), the fractions showing the ATP transport activity also contain the 56-kDa protein photolabeled with [γ-^{32}P]AB-ATP.

The results shown above strongly suggest that the 56-kDa polypeptide is, or at least is a component of, the ATP transporter in rat liver RER.

This report represents the first identification of the ATP transporter in rat liver RER, which is an important step toward its purification and molecular characterization. Peptide mapping of the photolabeled ATP transporter corresponding to the 56-kDa protein, purification, and preparation of antibodies to the purified ATP transporter shall be the subject of further studies, which eventually should lead to understanding the ATP transporter in rat liver RER.

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