Transport of Adenosine Triphosphate into Endoplasmic Reticulum Proteoliposomes†

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ABSTRACT: We have reconstituted a partially purified extract from rat liver endoplasmic reticulum membrane proteins into phosphatidylcholine liposomes. The resulting proteoliposomes, of an average diameter of 58 nm, transport intact ATP into their lumen in a temperature-dependent manner; transport was saturable (apparent K_m = 0.72 μM) and highly specific: CMP-sialic acid and GTP were transported very slowly or not at all. Transport of ATP was inhibited by DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid) but not by carboxyatractyloside. Previously, we showed that vesicles derived from rat liver and dog pancreas endoplasmic reticulum translocate ATP into their lumen *in vitro* but in these studies, following incubations with ATP, most of the phosphate was transferred to proteins because of the many kinases, endogenous acceptors for phosphorylation, and ATP binding proteins present in the vesicle membranes and lumen. This reconstituted system, which yielded a highly functional ATP transporter, can be used for further characterization and purification of this and probably other nucleotide transporters of the endoplasmic reticulum membrane. Previously used reconstitution protocols which were successful for Golgi membrane nucleotide transporters did not yield a functional endoplasmic reticulum ATP transporter.

Recent studies *in vitro* demonstrated an ATP transport activity in the membrane of the rough endoplasmic reticulum of mammals and yeast (Clairmont et al., 1992; Mayinger & Meyer, 1993).

The need for an ATP transporter in the ER[1] membrane had been hypothesized because ATP is synthesized primarily in mitochondria and must become available in the lumen of the ER for energy-requiring (Pfeffer & Rothman, 1987) and phosphorylation reactions (Hendershot et al., 1988; Leustek et al., 1991; Quemeneur et al., 1994). The former include dissociation of complexes between BiP and correctly folded and assembled proteins and peptides in the lumen of the ER (Munro & Pelham, 1986; Kassenbrock & Kelly, 1989; Dorner et al., 1990; Flynn, 1989), disulfide bond formation, and protein polymerization (Doms et al., 1987; Braakman et al., 1992). ATP is also the substrate for intralumenal phosphorylation of BiP (Hendershot et al., 1988; Leustek et al., 1991) and protein disulfide isomerase (Quemeneur et al., 1994).

Characterization and purification of the ER ATP transporter is necessary to (i) understand the mechanism of ATP translocation, (ii) determine the transporter arrangement within the rough ER membrane and its relationship with other ATP transporters such as those in mitochondria (Klingenberg, 1993) and the Golgi apparatus (Capasso et al., 1989) and (iii) determine whether the transport is a regulatory step and thereby affects the amount of ATP available in the lumen of the rough ER for the above described energy-requiring reactions. Because vesicles of the endoplasmic reticulum contain kinases, endogenous acceptors for phosphorylation and ATP binding proteins in their membranes and lumen, a reconstituted system of proteoliposomes which contains a functional ATP transporter must be developed. We have now done so and shown that the function mediated by this transporter is saturable, highly specific, and has different inhibitory behaviors compared to the mitochondrial ADP/ATP transporter.

EXPERIMENTAL PROCEDURES

Materials. The following radioactive compounds were purchased from Du Pont−New England Nuclear: [2,8-^{3}H]-ATP (28.8 Ci/mmol), [γ-^{35}S] ATP (1193 Ci/mmol), [8-^{3}H]-GTP (8.2 Ci/mmol), CMP-*N*-[9-^{3}H]sialic acid (21.2 Ci/mmol), L-α-dipalmitoyl[2-*palmitoyl*-9,10-^{3}H(N)]phosphatidylcholine (50.0 Ci/mmol), L-α-1-palmitoyl-2-oleoyl[*oleoyl*-1-^{14}C]phosphatidylcholine (52.6 mCi/mmol), sodium [^{3}H]-acetate (100.0 mCi/mmol), and deoxy-D-[1,2-^{3}H(N)]glucose (30.8 Ci/mmol).

Nonradioactive nucleotide derivatives, L-α-phosphatidylcholine (Type XI-E, from egg yolk), *n*-octyl β-D-glucopyranoside, and DEAE-Sephacel were purchased from Sigma Chemical Co. γS-ATP was purchased from Boehringer Mannheim and DIDS from Pierce Chemical Co.

Purification of Subcellular Fractions. Liver subcellular fractions were obtained from 150−200-g Sprague-Dawley male rats, which had been starved 18−20 h prior to decapitation with a guillotine. Endoplasmic reticulum-derived vesicles were obtained as previously described (Dallner, 1974). Vesicles were enriched 6-fold over cell homogenate in glucose-6-phosphatase specific activity (Arion, 1989) (21% yield), and 98% were sealed and "right side out" on the basis of latency of mannose-6-phosphatase activity (Arion, 1989). ER vesicles had 0.9-fold enrichment in sialyltransferase specific activity over cell homogenate (Carey & Hirschberg, 1981) (3% yield) and a 0.06-fold enrichment in succinate−cytochrome *c* reductase specific

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[1] Abbreviations: ER, endoplasmic reticulum, OG, *n*-octyl β-*O*-glucopyranoside, DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; PC, L-α-phosphatidylcholine; PMSF, phenylmethanesulfonyl fluoride; γS-ATP, adenosine 5′-*O*-(thio)triphosphate; BiP, binding protein; QELS, quasielastic light scattering.

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activity (Sottocasa et al., 1967) (0.2% yield). Mitochondria were isolated as described by Sordahl et al. (1971) and were enriched 3.2-fold, over cell homogenate specific activity in succinate−cytochrome *c* reductase (5% yield). From the succinate−cytochrome *c* reductase activity values in ER-derived vesicles and mitochondria, no more than 1% of the protein in the ER-derived vesicles fraction could have been mitochondria.

Endoplasmic Reticulum Protein Solubilization. ER proteins were resuspended at 3 mg/mL in 50 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 1 mM DTT, 1 mM PMSF, and 3% OG. The ratio of protein to detergent was 1:10 (w/w). The mixture was homogenized in a Dounce homogenizer using a Type B glass pestle and centrifuged at 100000*g* for 1 h at 2 °C.

DEAE-Sephacel Chromatography. The solubilized ER proteins were loaded onto a DEAE-Sephacel column (5.5 mg of protein/mL of gel) previously equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 10% glycerol, and 1 mM DTT) containing 1% OG. The column was washed with 3 volumes of buffer A plus 1% OG and then eluted at 0.5 mL/min with a linear gradient of 0−2 M NaCl in buffer A plus 1% OG. ATP transport activity was measured as described below using fractions eluted between 0.1 and 0.5 M NaCl.

Reconstitution of the ATP Transport Activity. Egg yolk PC was dried in glass ampoules as described by Milla and Hirschberg (1989) and resuspended at 4.0 mg/mL in buffer A containing 1% OG. The clear liquid solution was mixed with either solubilized ER proteins or proteins eluted from the DEAE column in a 6:1 PC to protein ratio (w/w). Protein was then incorporated into PC liposomes using a modification of the procedure described by Yu et al. (1989). Briefly, the PC and protein solution, kept on ice, was slowly diluted, at 1.5 mL/min, with continuous stirring with buffer A under a stream of nitrogen to a final concentration of OG of 0.15%. The reconstituted proteoliposomes were recovered as a pellet by ultracentrifugation at 200000*g* for 2 h at 2 °C and resuspended with a Dounce homogenizer in buffer A.

Translocation Assay. Transport of ATP into ER-derived vesicles and mitochondria was measured using the centrifugation assay previously described (Perez & Hirschberg, 1985). Vesicles and mitochondria (0.5−1.0 mg of protein) were incubated for 3 min at 30 °C in a final volume of 1 mL of buffer B (10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 1 mM $MgCl_2$) containing the desired concentration of ATP or other nucleotide derivatives. Following incubations, 2 mL of ice-cold buffer B was added to stop the reaction. The mixture was immediately centrifuged for 30 min at 100000*g* at 2 °C. The supernate was removed for calculations of $[S_m]$ (see below). The surface of the pellet was washed 4 times, each with 1.5 mL of buffer B. Ice-cold water (0.5 mL) was added to each pellet and mixed in a vortex mixer. Perchloric acid (8%; 0.5 mL) was added to each sample, which was transferred to an Eppendorf tube and placed on ice for 15 min. Following centrifugation in a microfuge for 5 min, the supernate was removed to calculate S_t (see below). To determine V_o and V_i (see below), sodium [^{3}H]acetate was used as a nonpenetrating standard and deoxy-D-[1,2-^{3}H(N)]glucose as a penetrating standard (Carey et al., 1980).

Transport into proteoliposomes was measured as previously described by Milla and Hirschberg (1989). Briefly, the reaction mixture contained 20−50 μg of protein as proteoliposomes, solutes whose transport was being measured, and sufficient buffer A for a final volume of 100 μL. Following incubation for 3 min at 30 °C, the mixture was loaded onto a 20- × 1-cm Sephadex G-50 column equilibrated with buffer A; the column was eluted at 0.3 mL/min with the same buffer. Fractions of 1 mL were collected. Solutes within proteoliposomes eluted in the void volume while free solutes eluted later. When it was necessary to discriminate between solutes within proteoliposomes and radioactivity transferred to protein, 1.5 mg of bovine serum albumin and trichloroacetic acid (7% final concentration) were added to each fraction. Following 10 min on ice, samples were centrifuged and the supernate was removed. The pellet, containing acid-insoluble radioactivity, was dissolved in NaOH (1 M; 1 mL) and reacidified with 0.4 mL of 4 M HCl.

Identification of Radioactive Solutes within Proteoliposomes and Incubation Medium. Radioactive solutes within proteoliposomes and incubation medium before and after incubations were determined as previously described by Capasso et al. (1989). Following transport assays, fractions excluded from the Sephadex G-50 columns (see above) were precipitated with perchloric acid (4% final concentration). The acid was removed from the supernate as previously described (Milla et al., 1992) and radiolabeled species were separated by HPLC using a Synchropak AX 100 column (SynChrom Inc.) at a flow rate of 2 mL/min using the following linear gradient: 0.05 M KH_2PO_4, pH 3.35 from 0 to 5 min; at 5 min, 0.60 M KH_2PO_4 increased to 0.75 M between 5 and 15 min and kept at 0.75 M between 15 and 25 min. The retention times were as follows: adenosine, 1.6 min; AMP, 6.0 min; ADP, 12.6 min; ATP, 18.4 min.

Calculations Used To Determine ATP Translocation. The calculations used in these experiments to measure ATP translocation are given below and as previously described (Perez & Hirschberg, 1987). $[S_m]$ = concentration of solutes in the incubation medium (micromolar) = (counts per minute per milliliter of solutes in the supernatant)/(specific activity of solute expressed as counts per minute per nanomole). S_t = total solutes in the pellet expressed in picomoles per milligram of protein = (total soluble radioactivity associated with the pellet, expressed as counts per minute per milligram of protein)/(specific activity of solutes, expressed as counts per minute per picomole). V_t = total pellet volume (microliters per milligram of protein) = volume outside (in between) vesicles + volume inside vesicles = (counts per minute per milligram of protein in the pellet for deoxyglucose)/(counts per minute per microliter of supernatant for deoxyglucose). V_0 = pellet volume which is outside (in between) vesicles (microliters per milligram of protein) = (counts per minute per milligram of protein in pellet for acetate)/(counts per minute per microliter of supernatant for acetate). V_i = pellet volume which is inside vesicles (microliters per milligram of protein) = $V_t - V_0$. S_0 = solutes which are outside (in between) vesicles in the pellet (picomoles per milligram of protein) = V_0 (microliters per milligram of protein) × $[S_m]$ (picomoles per microliter). S_i = solutes inside vesicles in the pellet (picomoles per milligram of protein) = $S_t - S_0$. $[S_i]$ = concentration of solutes inside vesicles in the pellet (micromolar) = S_i (picomoles per milligram of protein)/V_i (microliters per milligram of protein).

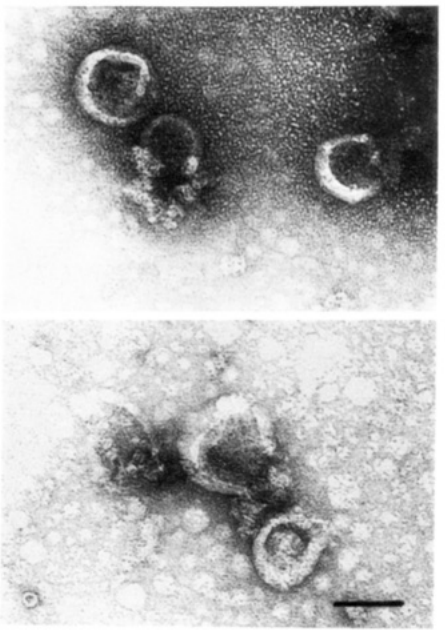

FIGURE 1: Electron microscopy of proteoliposomes fixed with glutaraldehyde and negatively stained with uranyl acetate. Bar = 50 nm.

RESULTS

Transport of ATP into Endoplasmic Reticulum Proteoliposomes Is Specific, Temperature-Dependent, and Saturable. Proteoliposomes were prepared from phosphatidylcholine and a mixture of proteins which were eluted from a DEAE-Sephacel column (see Experimental Procedures). Proteins had been solubilized from a highly purified endoplasmic reticulum membrane fraction (see Experimental Procedures). Examination of the proteoliposomes by electron microscopy, following negative staining with uranyl acetate, showed unilamellar vesicles ranging in size from 29 to 106 nm with an average diameter of 58 ± 18 nm (Figure 1); some vesicles in the preparation appeared irregular in shape, possibly an artifact induced by the staining process (not shown). The diameter of the same preparation of proteoliposomes determined by quasielastic light scattering (QELS) was 122 ± 31 nm. These values are in close agreement with previously reported ones for proteoliposomes (Milla & Hirschberg, 1989). The specific volume of the proteoliposomes calculated from electron microscopy was 3.99 μL/mg while the specific volume calculated from QELS was 6.93 μL/mg.

Either radiolabeled ATP, nucleotide sugars, or GTP was incubated with proteoliposomes and transport was determined by measuring the radioactivity which eluted in the void volume of a Sephadex G-50 column. As can be seen in Table 1, transport of ATP was highly specific; CMP-sialic acid which had previously been shown to be transported solely into the lumen of Golgi vesicles gave virtually no signal with the above proteoliposomes. In addition, a signal of less than 5% that of ATP was obtained with GTP a solute which enters ER vesicles very slowly or not at all. Transport of ATP was approximately 5-fold higher at 30 °C compared to 0 °C (9.71 ± 0.23 pmol mg^{-1} min^{-1} vs. 2.18 ± 0.95). Protein fractions eluted from the DEAE-Sephacel column at NaCl concentrations below 0.1 M or above 0.5 M showed specific ATP transport activities into proteoliposomes up to 25-fold lower than that of the fraction eluting between 0.1 and 0.5 M NaCl, which was used in the above studies.

Table 1: Transport of Nucleotide Derivatives into Endoplasmic Reticulum Proteoliposomes[a]

solutes	transport (pmol mg^{-1} min^{-1})
[2,8-^{3}H]ATP, 2 μM	8.8 ± 0.9
[γ-^{35}S]ATP, 2 μM	6.4 ± 2.0
[8-^{3}H]GTP, 2 μM	0.36 ± 0.03
CMP-[9-^{3}H]sialic acid, 5 μM	0.08 ± 0.05
UDP-[^{3}H]glucose, 5 μM	0.29 ± 0.01

[a] Solutes were incubated for 3 min at 30 °C with proteoliposomes as described in Experimental Procedures. Solutes within proteoliposomes eluted in the void volume of a Sephadex G-50 column. Results are average and standard error of 2−4 independent determinations.

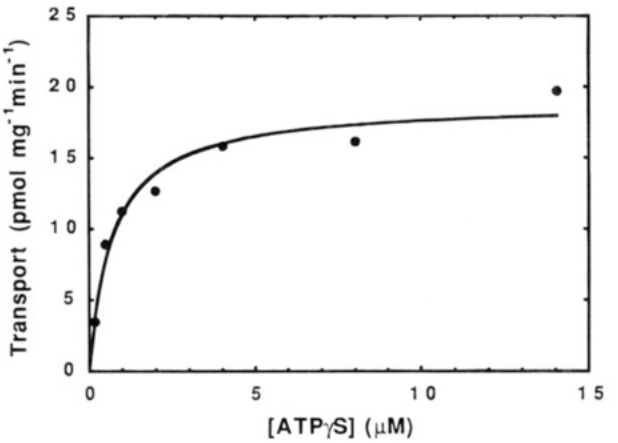

FIGURE 2: Rate of ATP transport into ER proteoliposomes versus concentration. Proteoliposomes (50 μg of protein) were incubated with different concentrations of [γ-^{35}S]ATP at a constant specific activity (3600 cpm/pmol) for 3 min at 30 °C. Transport was measured as described in Experimental Procedures. Points represent the mean of two separate determinations. The curve shows the least-squares fit of the Michaelis−Menten equation and the values for K_m and V_{max} were obtained using the Kaleida Graph software.

We next wanted to determine the affinity of the reconstituted ATP transporter. As can be seen in Figure 2, transport of ATP into proteoliposomes was saturable with an apparent K_m = 0.72 μM. The V_{max} was 18.9 pmol min^{-1} mg^{-1}. Controls were also performed to demonstrate that (a) endogenous lipids did not remain in the ER proteoliposomes and (b) exogenous phosphatidylcholine had actually been incorporated into these proteoliposomes. In the former case, radiolabeled phosphatidylcholine (3.5×10^6 cpm) was added to the OG-solubilized ER membrane extract which was subjected to DEAE-Sephacel chromatography; the proteoliposomes from the eluted proteins contained approximately 200 cpm, demonstrating that endogenous membrane lipids were not present in proteoliposomes. In the latter case, radiolabeled phosphatidylcholine was added as a tracer to the DEAE-Sephacel eluted protein along with egg yolk phosphatidylcholine solubilized in OG-containing buffer (see Experimental Procedures). From the amount of tritiated phosphatidylcholine incorporated into proteoliposomes, we can calculate that the resulting proteoliposomes had a ratio of phosphatidylcholine to protein of 1.61 ± 0.30 μmol/mg, very similar to the ER protein translocator system reconstituted previously (Yu et al., 1989).

Intact ATP Enters Endoplasmic Reticulum Proteoliposomes. To determine whether intact ATP entered the lumen of ER proteoliposomes, these were incubated with [^{3}H]ATP for 4 min. We then determined, by HPLC, the radioactive solutes within proteoliposomes and incubation medium. As

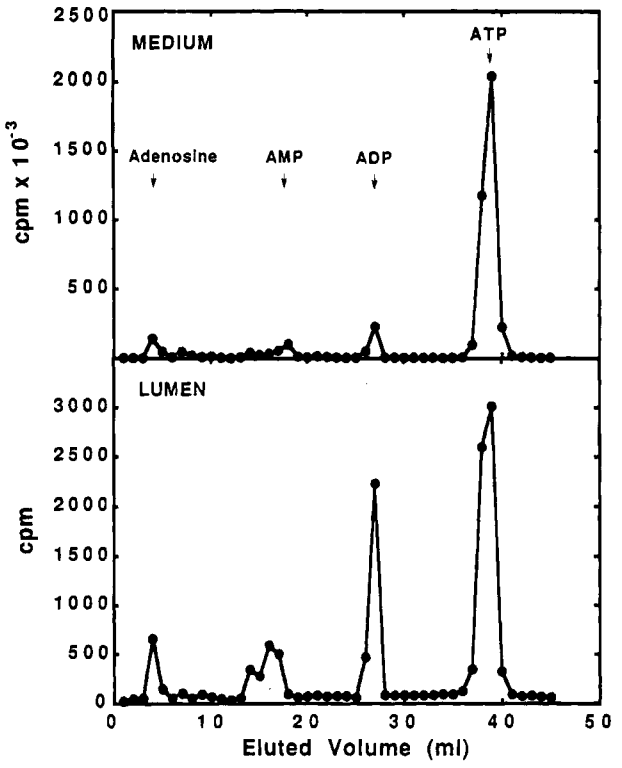

FIGURE 3: Analysis of radiolabeled solutes following [^{3}H]ATP transport into ER proteoliposomes. Proteoliposomes were incubated for 4 min with 2 μM [^{3}H]ATP at 30 °C. An aliquot of the incubation medium was subjected to HPLC analysis as described in Experimental Procedures. Another aliquot was subjected to Sephadex G-50 chromatography and solutes of excluded material (proteolipsomes) analyzed as described above. Arrows indicate elution of nonradioactive standards.

shown in Figure 3, ATP was the major solute of both compartments, demonstrating that intact ATP enters the lumen of proteoliposomes. In addition, the lumen of proteoliposomes contained significantly more ADP, AMP, and adenosine than the incubation medium (Figure 3). This is most likely the result of kinases, ATPases, phosphatases, and other hydrolytic enzymes oriented toward the lumenal side of proteoliposomes having longer time to act upon ATP because it takes longer to analyze the lumenal content of proteoliposomes (a Sephadex G-50 column is required) than is required for the incubation medium.

Characterization of ATP Transport into Endoplasmic Reticulum Proteoliposomes. It was important to determine the behavior of the reconstituted ER transporter toward inhibitors such as DIDS and carboxyatractyloside. As can be seen in Table 2, DIDS inhibited transport of ATP into ER proteoliposomes and ER vesicles to a very similar extent. Carboxyatractyloside, on the other hand, inhibited ATP transport into ER proteoliposomes only slightly, consistent with minor contamination of this preparation fraction with mitochondria. The fact that carboxyatractyloside caused significant inhibition of ATP transport into mitochondria, as expected (Table 2), demonstrates that the lack of inhibition observed with ER proteoliposomes and ER vesicles was not due to inactive glycoside. Together, these results and those previously shown in Table 1 and Figure 1 strongly suggest that ATP transport characteristics of the reconstituted proteoliposomes are very different from mitochondria and consistent with an ATP transport activity endogenous to ER membranes.

An additional expected but important difference in the behavior of ATP translocation into endoplasmic reticulum proteoliposomes and endoplasmic reticulum vesicles was that with proteoliposomes most of the ATP (and phosphate) was found to be intact and soluble in the lumen, while in vesicles, the majority of the phosphate derived from ATP (approximately 80%) was membrane-bound, with the remainder being soluble within the vesicles' lumen. The DEAE-Sephacel column recovered ER kinases, high-affinity ATP binding proteins, and endogenous acceptors of phosphorylation from the ATP transporter; thus in the partially purified proteoliposomes only 20% of radiolabeled phosphate was membrane-bound while the remainder was soluble within the lumen. One would expect that with the purified ATP transporter in liposomes, all radioactivity derived from ATP will be soluble within the lumen of these liposomes.

DISCUSSION

We have functionally reconstituted and characterized the ATP transporter of the rat liver endoplasmic reticulum membrane into phosphatidylcholine liposomes. This approach was then used for the initial purification of this translocator protein.

An important difference was found between the functional reconstitution approach used in this study and that used previously with Golgi membrane nucleotide sugar and nucleotide sulfate transporters (Milla & Hirschberg, 1989; Milla et al., 1992; Mandon et al., 1994). The endoplasmic reticulum ATP transporter was not functional following the freeze–thaw cycles prior to incorporation into phosphatidylcholine liposomes as described for the Golgi nucleotide transporters. Attempts to use this latter protocol with several detergents and endogenous lipids from the endoplasmic reticulum to functionally reconstitute the endoplasmic reticulum ATP transporter were also unsuccessful. The reason

Table 2: Inhibition and Mitochondria by DIDS and Carboxyatractyloside of ATP Transport into Endoplasmic Reticulum Vesicles and Proteoliposomes[a]

	ATP transport (pmol mg^{-1} min^{-1})			
	ER vesicles	proteoliposomes	bovine heart mitochondria	rat liver mitochondria
none	11.22 ± 2.18	13.74 ± 2.61	79.61 ± 2.18	39.50 ± 1.11
DIDS	3.46 ± 0.14	2.78 ± 0.81	1.76 ± 0.60	13.47 ± 0.30
carboxyatractyloside	10.73 ± 1.42	12.48 ± 2.33	16.80 ± 2.89	26.95 ± 1.51

[a] ER membranes (500 μg of protein), DEAE-eluted protein (20 μg) in proteoliposomes, and rat liver or bovine heart mitochondria (250 μg of protein) were incubated with 2 μM ATPγS. In the carboxyatractyloside inhibition experiments, the samples were preincubated for 5 min at 0 °C in the presence of 5 μM carboxyatractyloside followed by 5 min at 30 °C. The activity was assayed as described in Experimental Procedures. In the DIDS inhibition experiments, the samples were preincubated for 5 min at 0 °C with 0.4 nmol of DIDS/μg of protein and the activity was measured as before.

for this unexpected behavior of the endoplasmic reticulum ATP transporter is not known.

The reconstituted ATP translocator was highly specific regarding substrates and showed an affinity for ATP somewhat higher than the endoplasmic reticulum vesicles (Clairmont et al., 1993). This is mot likely the result of the absence of kinases, endogenous phosphorylation acceptors, and ATP binding proteins in the partially purified proteins used in the reconstitution. The inhibition of DIDS and absence of inhibition by carboxyatractyloside further demonstrate the functionality of the transporter and the absence of significant contamination by the ADP/ATP transporter of the mitochondria. The difference in the size of the proteoliposomes as determined by electron microscopy or QELS is not surprising as the two techniques measure size based on different principles and therefore have different limitations. QELS is based on analyzing fluctuations of scattered light intensity caused by particles in solution undergoing Brownian motions; this yields information about the translational diffusion coefficient and the corresponding Stokes–Einstein diameter of the particle. The scattering capability of a particle is strongly dependent on its diameter, which therefore influences the results when describing a polydispersed system such as in this case (Elorza et al., 1993; Chen et al., 1976). On the other hand, negative staining is known to cause some vesicle shrinkage and shape distortion (Arrio et al., 1974). Nevertheless, the specific volumes of the proteoliposomes were quite similar when determined by either centrifugation in the presence of penetrators and nonpenetrators or as calculated by EM and QELS.

This is the first nucleotide transporter of the endoplasmic reticulum membrane which has been reconstituted into liposomes; except for the bile acid transporter (Dippe & Levy, 1993), no other endoplasmic reticulum membrane transporter has been reconstituted to date. Recently the nascent protein translocation system (Gorlich & Rapoport, 1993) and several inorganic cation and proton channels of the endoplasmic reticulum membrane have been reconstituted into proteoliposomes (Vassilev et al., 1987; Gluck & Caldwell, 1988).

The mechanism for ATP translocation into the endoplasmic reticulum lumen is not known, although by analogy with nucleotide sugar transporters it may be an antiporter with ADP or AMP (Clairmont et al., 1993; Capasso et al., 1989). In the Golgi apparatus, preliminary evidence suggest that ATP enters via an AMP antiporter (Capasso et al., 1989).

The above described reconstitution approach should allow us to further purify the transporter, to determine whether the antiporter mechanism is operative in this instance, and to determine the nature of the exiting molecule. In addition, peptide sequence obtained from the purified transporter will allow its cloning. This will enable us to understand how the transporter is arranged in the membrane, its relationship to the mitochondria and Golgi ATP transporters, and whether *in vivo* it regulates the amount of ATP available in the lumen of the endoplasmic reticulum for energy-requiring and phosphorylation reactions.

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