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An ATP transporter is required for protein translocation into the yeast endoplasmic reticulum

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The transfer of precursor proteins through the membrane of the rough endoplasmic reticulum (ER) in yeast is strictly dependent on the presence of ATP. Since Kar2p (the yeast homologue of mammalian BiP) is required for translocation, and is an ATP binding protein, an ATP transport system must be coupled to the translocation machinery of the ER. We report here the characterization of a transport system for ATP in vesicles derived from yeast ER. ATP uptake into vesicles was found to be saturable in the micromolar range with a K_m of 1×10^{-5} M. ATP transport into ER vesicles was specifically inhibited by 4,4'-diisothiocyanatostilbene -2,2'-disulfonic acid (DIDS), a stilbene derivative known to inhibit a number of other anion transporters, and by 3'-*O*-(4-benzoyl)benzoyl-ATP (Bz_2-ATP). Inhibition of ATP uptake into yeast microsomes by DIDS and Bz_2-ATP blocked protein translocation *in vitro* measured co- as well as post-translationally. The inhibitory effect of DIDS on translocation was prevented by coincubation with ATP. Moreover, selective membrane permeabilization, allowing ATP access to the lumen, restored translocation activity to DIDS-treated membranes. These results demonstrate that translocation requires a DIDS and Bz_2-ATP-sensitive component whose function is to transport ATP to the lumen of the ER. These findings are consistent with current models of protein translocation in yeast which stipulate the participation of Kar2p in the translocation process.

Key words: ATP transport/endoplasmic reticulum/protein translocation/*Saccharomyces cerevisiae*

Introduction

The first step in the transport of secretory proteins to the cell surface is their translocation across the membrane of the endoplasmic reticulum (ER) (Meyer, 1991). In yeast, where translocation can be uncoupled from translation, a requirement for ATP in the translocation process has been found (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986b; Waters and Blobel, 1986). Moreover, using a cytosol-free *in vitro* assay (Sanz and Meyer, 1988), events occurring at the membrane could be examined and two discernible steps of translocation were defined. The first step, the binding of precursor proteins to the ER membrane, was found to be ATP-independent. The second event, the transfer of the bound preproteins across the membrane, is strictly dependent on the presence of ATP (Sanz and Meyer, 1989). Although a number of cytosolic and membrane components have been identified that are required for recognition, targeting and processing of preproteins (Rapoport, 1990; Meyer, 1991), the connection between ATP hydrolysis and preprotein transport is poorly understood. Major unanswered questions concern the site of action and the role of ATP in the translocation process.

Protein translocation in yeast involves the participation of at least one luminal ATP binding ER protein. Mutants with defects in or reduced expression of the *KAR2* gene, a homolog of mammalian luminal protein BiP, show impaired protein translocation (Normington *et al.*, 1989; Rose *et al.*, 1989; Vogel *et al.*, 1990; Nguyen *et al.*, 1991). Kar2p has been shown to be required in the translocation step for the ATP-dependent interaction of preproteins with the *SEC61* gene product (Sanders *et al.*, 1992). Based on the fact that BiP binds ATP, and that it associates and dissociates from proteins in an ATP-dependent manner (Gething and Sambrook, 1990), Kar2p represents a potential link between ATP hydrolysis and protein translocation. The maintenance of sufficient ATP levels in the ER lumen would be a prerequisite for Kar2p's participation. This implies the existence of an ATP transporting system to supply the luminal Kar2p with ATP from the cytosol. Accordingly, experiments were designed to identify such an activity and to examine its participation in the translocation process. In the studies reported here, we have characterized an ATP transporting activity in membranes derived from the endoplasmic reticulum of *Saccharomyces cerevisiae*. Most importantly, the selective inhibition of this activity shut down all modes of protein translocation *in vitro*.

Results

Kinetic properties of the yeast microsomal ATP transporter

We first ascertained that rough microsomes derived from *S.cerevisiae* possess an ATP transporting activity. For measuring ATP uptake, an ion exchange filtration assay was developed (see Materials and methods). This assay was suitable for resolution of the initial velocity of uptake. Results from these experiments indicated that uptake was linear within the first 30 s over the concentration range studied (not shown). For measuring the concentration dependence of ATP transport into yeast rough microsomes, the uptake within the first 20 s was determined and the external ATP concentration was varied from 1 to 100 μM (Figure 1A). In order to preclude the influence of any mitochondrial contamination, carboxyatractyloside, a potent specific inhibitor of the mitochondrial ADP/ATP carrier (Klingenberg, 1985), was present in all uptake reactions. From the reciprocal plot shown in Figure 1B, ATP uptake has an apparent K_m of 10.1 μM and a V_{max} of 1.2 μM/min *g* protein. This K_m value is similar to that of the mitochondrial ADP/ATP transporter for which precise measurements have been made (for review see Klingenberg, 1985). As can be deduced from

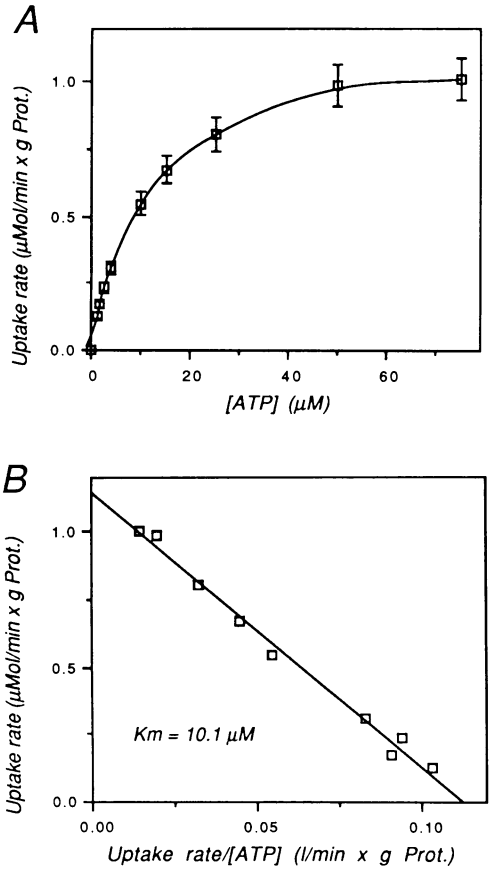

Fig. 1. ATP uptake into yeast microsomes. (**A**) Initial velocity analysis of ATP uptake. Equilibration kinetics were measured using [α-^{32}P]ATP. The transport assay was carried out in the presence of 100 μM carboxyatractyloside to suppress uptake due to mitochondrial contaminants. Only single time points, obtained within the first 20 s, were measured to obtain the concentration dependence of uptake. In control experiments it was proven that the initial 30 s of kinetics were within the linear range. For determining specific uptake, the amount of ATP associated with octyl glucoside-permeabilized membranes was subtracted. Data are from four individual sets of uptake experiments. (**B**) Reciprocal plot of kinetic data according to Eadie–Hofstee. The mean values shown in Figure 1A were used for the calculations. The kinetic constants were evaluated by linear regression of the reciprocal plots.

the reciprocal plot of uptake kinetics (Figure 1B), a single uptake system was characterized over the concentration range studied. This result also rules out a rate-limiting effect of a luminal counter substrate. Preliminary results suggest that ATP uptake may be coupled to an export of AMP in a non-rate-limiting fashion (not shown).

ATP transport requires intact microsomes and is inhibited specifically by DIDS

Having established the existence of an ATP transporting activity, we next examined specific inhibitors whose effect on ATP transport and protein translocation could be studied. ATP uptake was only minimally sensitive to carboxyatractyloside (Figure 2A). This small amount of inhibition was most likely related to a slight residual contamination by functional mitochondria. In contrast to ATP uptake into mammalian microsomes (Clairmont *et al.*, 1992), ATP transport into yeast microsomes was essentially resistant to proteolysis by proteinase K and trypsin. The amount of ATP taken up by rough microsomes was decreased considerably

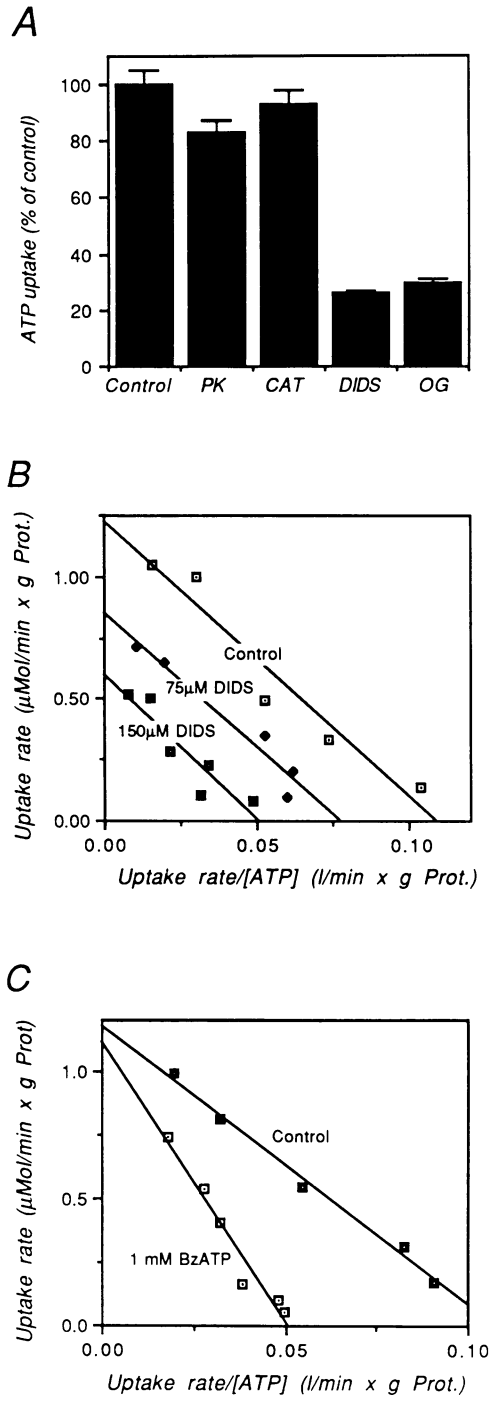

Fig. 2. Inhibition of microsomal ATP transport. The ATP associated with microsomes was measured using the uptake assay described in Materials and methods. Data are from three independent measurements. (**A**) Membranes were pretreated as follows: control, mock-treated microsomes (15 min, 25°C); PK, 0.5 mg/ml proteinase K (60 min, 0°C); CAT, 100 μM carboxyatractyloside (15 min, 25°C); DIDS, 1 mM 4,4′-diiso-thiocyanatostilbene-2,2′-disulfonic acid (15 min, 25°C); OG, 0.5% octyl glucoside (15 min, 25°C). (**B**) Inhibition of ATP transport by modifications of membranes with DIDS. Kinetic data are depicted according to Eadie–Hofstee. Membranes were incubated with DIDS at the indicated concentrations for 15 min at 25°C and reisolated, before ATP uptake was measured as in Figure 1. (**C**) Competitive inhibition of ATP transport with Bz_2ATP. Kinetic data are depicted according to Eadie–Hofstee. Membranes were incubated with 1 mM Bz_2ATP for 5 min at 0°C in the dark. ATP transport was measured as in Figure 1 under dimmed light.

when the membranes were permeabilized with 0.5% *n*-octyl β-D-glucopyranoside (octyl glucoside). Using immunoblotting techniques we found that treatment with 0.5% octyl glucoside at this concentration released Kar2p from the membranes but did not solubilize the integral membrane protein Sec61p (not shown). The sensitivity of the assay to octyl glucoside argues that ATP transport, not binding, is being measured.

Significant inhibition of ATP uptake was obtained by pretreatment of the membranes with the membrane-impermeable reagent DIDS. This compound is a well-characterized inhibitor of the anion transporter of erythrocytes (Cabantchik and Rothstein, 1974; Passow, 1986). The low level of DIDS-resistant ATP association with microsomes equalled the level of ATP bound to detergent-permeabilized membranes and was therefore considered to be unrelated to transport. The effect of DIDS on specific, i.e. 0.5% octyl glucoside-sensitive, ATP uptake is shown in Figure 2B. Based on the Eadie−Hofstee presentation of these data, DIDS inhibition of ATP transport is non-competitive. This finding is in accordance with reports that show that DIDS irreversibly inactivates anion transporters by reacting with amino acids in the active site (Passow, 1986). A DIDS concentration of 150 μM resulted in a half-maximal inhibition (IC_{50}) of ATP transport.

ATP transport is also inhibited by an ATP analogue

We examined the ability of two different ATP analogues, 8-azido-ATP and Bz_2-ATP to competitively inhibit ATP transport under conditions where covalent crosslinking was avoided (dim or no light). Although both of these analogues have been effectively employed to photoaffinity label certain nucleotide binding sites (Czarnecki, 1979; Williams and Coleman, 1982), treatment of yeast microsomes with 8-azido-ATP, at concentrations as high as 1 mM, had little effect on ATP transport (not shown). In contrast, incubation with Bz_2-ATP (in the dark), competitively reduced ATP uptake into microsomes with a K_i of 850 μM (Figure 2C). The poor affinity of 8-azido-ATP for the microsomal ATP transporter may well be due to the fact that, in this analogue, a *syn* conformation of the purine with respect to the ribose is the most stable one, whereas Bz_2-ATP, like ATP itself, has these groups in the preferred *anti* conformation. Similarly, the mitochondrial ADP/ATP carrier shows a higher affinity for nucleotides in an *anti* conformation, and accordingly, 8-azido-ATP is a relatively weak ligand (Schäfer *et al.*, 1976; Mayinger *et al.*, 1989). Using the nucleotide uptake assay described in Materials and methods, we found that neither of these ATP analogues were transported by the microsomal ATP uptake system, which again is comparable with the mitochondrial transport system (Klingenberg, 1985). Fortunately, we were able successfully to inactivate ATP transport into yeast microsomes by UV-induced crosslinking by Bz_2-ATP (see below).

Inhibition of ATP transport with DIDS inhibits preprotein translocation

The DIDS sensitivity of rough microsomal ATP transport allowed us to test whether ATP transport is required for preprotein translocation. To facilitate quantification of translocation, a novel unglycosylated form of the yeast presecretory protein prepro-α-factor was constructed. The three oligosaccharide-accepting asparagine residues were replaced with glutamines. In contrast to conventional prepro-α-factor, where variable glycosylation yields several forms of translocated pro-α-factor in *in vitro* reactions (Hansen *et al.*, 1986), the unglycosylated prepro-α-factor form results in only a single translocated species. Treatment of membranes with increasing concentrations of DIDS inactivated the translocation of prepro-α-factor (Figure 3A). Translocation dropped to background levels at 1 mM DIDS. These data suggest that translocation is dependent on a functional ATP transporting system.

Three different yeast cell-free assays are routinely used to study protein translocation. These include: a cotranslational assay, where membranes are present during the biosynthesis of the preprotein (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986a; Waters and Blobel, 1986); a post-translational assay, in which cycloheximide is added to stop translation prior to the addition of membranes to the lysate (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986b; Waters and Blobel, 1986); and a cytosol-free assay, where chemically pure radiolabeled preprotein is diluted out of denaturant into an aliquot of microsomal membranes in buffer (Sanz and Meyer, 1988, 1989; Bush *et al.*, 1991). These latter two assays, in which translocation can be uncoupled from translation, have been used extensively to demonstrate a requirement for ATP in the translocation reaction (Hansen *et al.*, 1986; Rothblatt and Meyer, 1986b; Waters and Blobel, 1986; Sanz and Meyer, 1989).

Our results show that DIDS inactivation of ATP transport inhibited translocation in all three *in vitro* assays. As shown in Figure 3B−D, virtually identical DIDS concentrations were required for half-maximal inhibition of co- and post-translational translocation in yeast lysates (IC_{50} = 460 and 480 μM respectively) as well as the translocation of prepro-α-factor diluted out of urea in a cytosol-free system (IC_{50} = 490 μM). This implies that the same DIDS-sensitive factor is required for both co- and post-translational modes of translocation. The DIDS inactivation of translocation paralleled that of the DIDS inactivation of ATP transport, although lower levels of DIDS were needed for half-maximal inhibition of ATP transport (compare the curve in Figure 3E with curves 3B−D). A plausible explanation for this finding is that translocation is regulated by a critical ATP concentration in the ER lumen, rather than ATP transport as a rate-limiting step (see also Figure 5).

It could be argued that the DIDS inhibition of translocation is not affiliated with its ability to inhibit ATP transport. For that reason it was necessary to characterize the DIDS effect on translocation in greater detail. Consistent with our postulation that ATP transport is connected with preprotein translocation would be the ability of ATP itself to competitively block the DIDS inhibition of translocation. This would directly implicate ATP binding proteins as the target of DIDS inactivation. Shown in Figure 4 are the results of experiments where membranes were coincubated with DIDS and various amounts of ATP. ATP efficiently protected the preprotein translocation competence of microsomes from inactivation by DIDS. This provides additional evidence linking ATP transport and translocation, as well as indicating that the DIDS-sensitive component is an ATP binding protein. The protection by ATP is specific as ADP was considerably less effective (not shown).

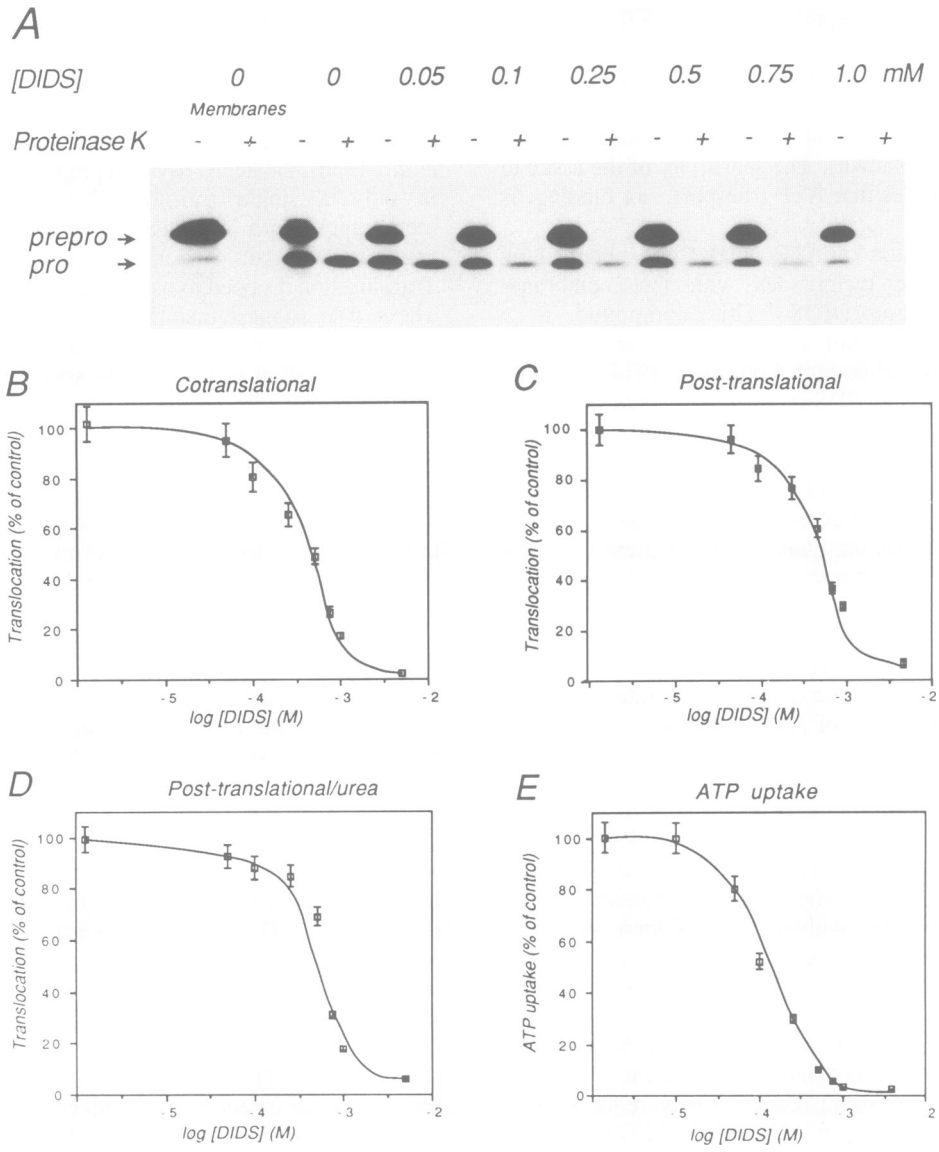

Fig. 3. DIDS inhibits prepro-α-factor translocation into yeast microsomes. **(A)** Inhibition of post-translational translocation. Translocation was measured using a prepro-α-factor construct lacking the three glycosylation sites as described in Materials and methods. The fluorogram shows the effect of increasing DIDS concentrations on post-translational translocation. Translocation was assayed by protection of pro-α-factor from digestion by proteinase K. Treatment of membranes with DIDS was performed as described in Materials and methods. Concentrations of DIDS as well as the presence or absence of protease is shown above the figure. Prepro-, prepro-α-factor; pro, pro-α-factor. Membranes were present in all assays unless otherwise indicated. (B–D) Quantification of DIDS inhibition of co- and post-translational translocation of prepro-α-factor using Geiger densitometry. Data are from three independent translocation experiments, respectively. **(B)** Co-translational translocation; **(C)** post-translational translocation; **(D)** cytosol-free translocation of purified ^{35}S-labeled prepro-α-factor)$(His)_6$ by dilution out of urea. **(E)** Titration curve of DIDS inhibition of ATP uptake into yeast microsomes. Membranes were incubated with DIDS (0.01–5 mM) for 15 min at 25°C and reisolated, before ATP uptake was measured according to Materials and methods using 50 μM [α-^{32}P]ATP. For the determination of ATP transported into the lumen of the vesicles, the amount of ATP associated with octyl glucoside-permeabilized membranes was subtracted.

Inhibition of ATP transport by photocrosslinking with Bz_2-ATP also inhibits translocation

If the interpretation made above is valid, the same results should be obtained using an inhibitor of ATP uptake other than DIDS. We therefore irradiated microsomes with UV light in the presence of increasing amounts of Bz_2-ATP and determined the effect on preprotein translocation (Figure 5A). Control experiments showed that UV light alone had no effect on translocation activity (not shown). A concentration of 2.5 mM Bz_2-ATP was found to be required to reduce translocation to half-maximal activity, whereas only 0.9 mM was required for the half-maximal inhibition of ATP uptake (Figure 5B). This is similar to what was observed in the case of DIDS inhibition, where ATP transport was reduced significantly before a clear effect on translocation was observed.

The results presented above suggest that an ATP binding protein, sensitive to DIDS and Bz_2-ATP, functions in preprotein translocation by supplying luminal components with needed ATP. It cannot be unequivocally ruled out that a different ATP binding protein, located on the cytosolic side of the membrane, is the DIDS and Bz_2-ATP-sensitive component that functions in translocation. We therefore analyzed in greater detail the action of both inhibitors on the translocation of preproteins in the cytosol-free assay (Sanz and Meyer, 1988). The results obtained for the

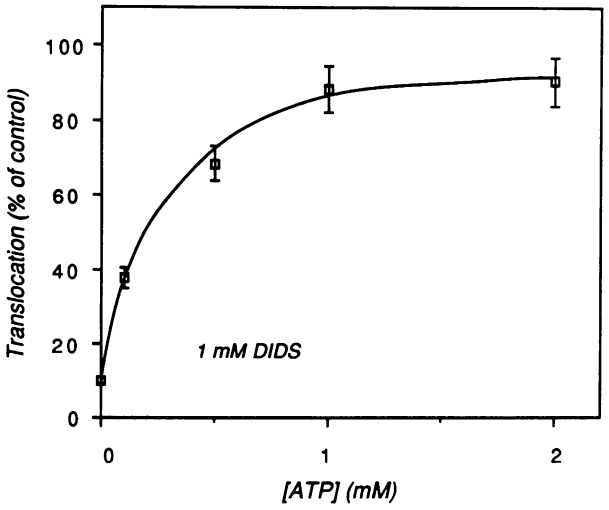

Fig. 4. ATP protects microsomes from DIDS inactivation. Membranes coincubated with 1 mM DIDS and varying amounts of ATP were re-isolated and added to an assay measuring the cytosol-free translocation of purified ^{35}S-labeled prepro-α-factor(His)$_6$ by dilution out of urea. Translocation was quantified by radioanalytic imaging and expressed as the ratio of protease-resistant forms of prepro-α-factor to total prepro-α-factor added to the reaction. Control translocation assays were carried out on mock-treated membranes.

inhibition of translocation using radiochemically pure preprotein diluted out of 8 M urea into the membrane sample were reanalyzed and depicted as semi-reciprocal plots (Figure 6). Two distinct phases of DIDS (Figure 6A) and Bz_2-ATP-induced inhibition (Figure 6B) of translocation were obtained. The region of the curves corresponding to low inhibitor concentrations show only very minor effects on preprotein translocation. This was followed by a region of linearly increasing inhibition at higher concentrations. Such behavior is typical for the inhibition of an enzyme catalyzing a non-rate-limiting step in a multi-component system (Rognstad, 1979). When the reaction is inhibited to a critical extent, it becomes rate limiting and a linear reduction of the overall flux, in our case preprotein translocation, is observed. Treatment of microsomes with 0.5 mM DIDS and 2 mM Bz_2-ATP respectively (corresponding to an ~80% reduction of ATP transport activity) represents the transition to this sharp reduction of translocation. These findings provide additional evidence that ATP transport is indeed connected with preprotein translocation, but is not rate-limiting for this process. This allows an inhibition of ATP transport up to a critical level without a significant effect on translocation. Beyond this point, however, translocation decreased rapidly.

Translocation activity of DIDS-treated membranes can be partially restored by limited permeabilization with octyl glucoside

A direct proof showing that DIDS inactivates necessary ATP transport, and not a different ATP binding protein required for translocation, could be generated by demonstrating that translocation can occur in DIDS-treated membranes by physically supplying the luminal components with ATP. Microsomes could be 'loaded up' with ATP, and then poisoned with DIDS to knock out transport prior to removal of ATP in the medium. If residual translocation activity were observable, one could conclude that the luminal pool of ATP

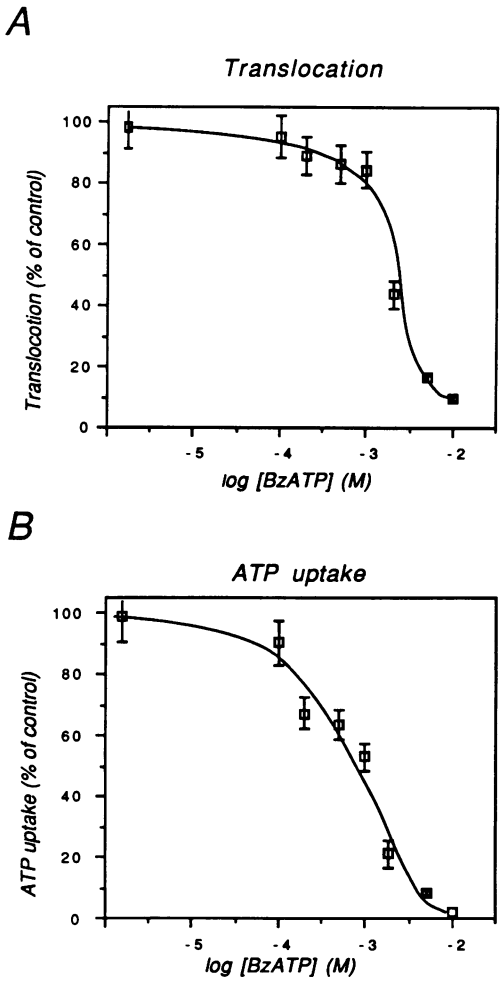

Fig. 5. Photocrosslinking with Bz_2-ATP inhibits prepro-α-factor translocation and ATP transport into yeast microsomes. (**A**) Inhibition of translocation. Cytosol-free translocation of purified ^{35}S-labeled prepro-α-factor(His)$_6$ by dilution out of urea was measured. Data are from two independent translocation experiments. Membranes were irradiated for 5 min with different concentrations of Bz_2-ATP and reisolated as described in Materials and methods. Translocation was performed as in Figure 3. (**B**) Inhibition of ATP uptake. Aliquots of the same membranes used in (A) were assayed for ATP transport activity according to Materials and methods using 50 μM [α-^{32}P]ATP.

is sufficient to mediate translocation in the absence of a functioning transporter. Such a study was not possible, as luminal ATP was hydrolyzed too rapidly during the time required to treat the membranes with DIDS and remove ATP from the medium (data not shown).

Instead, we chose a different, but feasible, approach in which DIDS-inactivated membranes were permeabilized with sub-solubilizing amounts of detergents in order to allow ATP to diffuse into the lumen and restore translocation activity. It has recently been shown that treatment of liposomes with amounts of detergents far below their CMC results in an increase in permeability to protons or molecules such as 6-carboxyfluorescein (mol. wt = 376) (Lichtenberg, 1985; Paternostre *et al.*, 1988). Appropriate octyl glucoside concentrations, defined as those that would not allow proteolytic digestion of translocated preproteins, were determined to lie in the range of 0.02−0.05% (not shown). Although DIDS-treated membranes show very low levels of residual translocation activity, within this range of detergent concentrations, significant increases in the amount

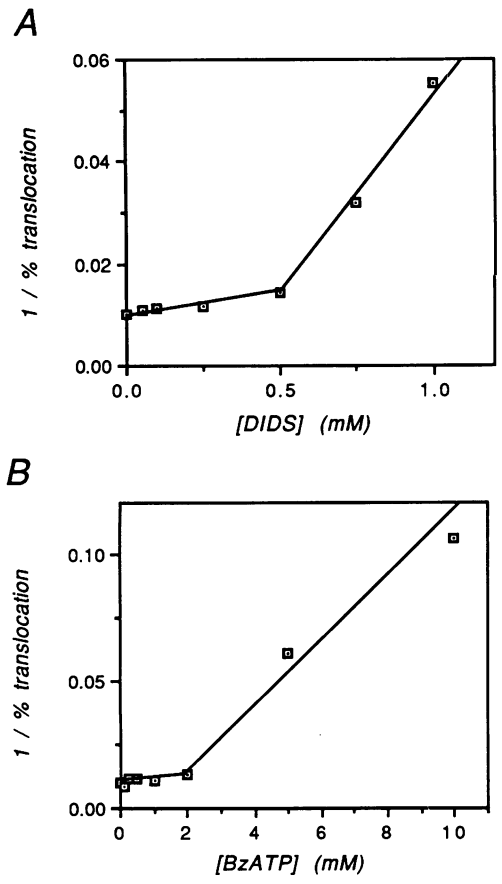

Fig. 6. DIDS and Bz_2-ATP do not inhibit a rate limiting step of prepro-α-factor translocation in yeast. Semireciprocal presentation of DIDS and Bz_2-ATP inhibition of translocation. Cystol-free translocation of purified ^{35}S-labeled prepro-α-factor(His)$_6$ by dilution out of urea was measured. (**A**) Membranes were treated with DIDS as in Figure 3. (**B**) Membranes were treated with Bz_2-ATP as in Figure 5.

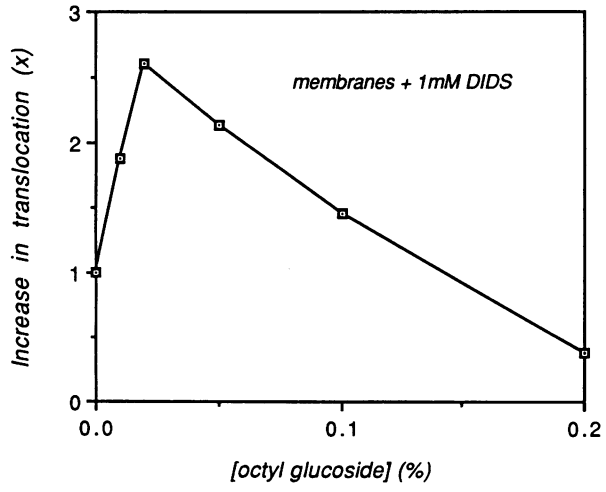

Fig. 7. Selective permeabilization of membranes with octyl glucoside increases the translocation activity of DIDS-inactivated membranes. Post-translocational translocation of prepro-α-factor was measured using membranes pretreated with 1 mM DIDS and subsequently permeabilized with increasing concentrations of octyl glucoside. The amount of proteinase K protected signal cleaved pro-α-factor was quantified by Geiger densitometry. The increase in the amount of protected pro-α-factor over background after octyl glucoside treatment is depicted.

of protease protected pro-α-factor were observed (Figure 7). Octyl glucoside treatment of control membranes had no stimulatory effect on translocation (not shown). A loss of protease protection was observed at higher concentrations of octyl glucoside indicating that the membranes had been rendered permeable to macromolecules. Direct measurements of the amount of ATP associated with permeabilized microsomes is impossible as rapid diffusion of ATP occurs during the isolation procedure. Nevertheless, these results represent a more direct demonstration that the access of ATP to the lumen of yeast ER is part of the process of preprotein transport across the ER membrane in yeast, and that reagents such as DIDS inhibit this necessary transport.

Discussion

The data presented here indicate that yeast ER possesses an ATP transport system whose kinetic analysis defines it as an enzyme-mediated process. The derived K_m (Figure 1) is similar to that of the mitochondrial ADP/ATP carrier, a well-characterized nucleotide transporter (Klingenberg, 1985). The kinetic analysis is consistent with the presence of a single uptake system in the microsomal preparation. ATP uptake was found to be sensitive to DIDS (Figure 2A and B), a well-characterized inhibitor of anion transporters related to the type found in erythrocytes (Cabantchik and Rothstein, 1974; Passow, 1986). DIDS has recently been reported also to inhibit a number of other anion transporters, including the antiport of glutamate and aspartate in mitochondria (Diercks *et al.*, 1992), transport of nucleotide sugars and ATP into Golgi (Capasso and Hirschberg, 1984) and the uptake of ATP into mammalian rough microsomes (Clairmont *et al.*, 1992). The photoaffinity nucleotide analog Bz_2-ATP, which also inhibited ATP transport into microsomes (Figure 2C), has been used previously to inactivate the mitochondrial F_1-ATPase (Williams and Coleman, 1982).

Inhibition of ATP transport with DIDS blocked all modes of preprotein translocation (Figure 3A–D). Our data do not completely eliminate the possibility that DIDS affects ATP uptake and preprotein translocation in a similar yet independent manner. As mentioned above, DIDS is a potent inhibitor of anion transport. Recent studies indicate that a putative protein translocating channel in mammalian ER can be characterized by its ability to transport an anion, i.e. glutamate (Simon and Blobel, 1991). One could therefore postulate that such a channel, rather than an ATP transporter, is the DIDS-sensitive component of the translocation process. Strong evidence linking the DIDS effect on preprotein translocation directly to its effect on ATP transport is provided, however, by our finding that the translocation-inhibiting activity of DIDS was efficiently prevented by coincubation with ATP (Figure 4). This is in line with published data on other DIDS-sensitive anion transporters, such as the mitochondrial aspartate/glutamate carrier (Diercks *et al.*, 1992) and the anion transporter from erythrocytes (Cabantchik and Rothstein, 1974; Passow, 1986). In these cases, as in ours, coincubation with substrate prevented inhibition when the substrate binding site was covalently modified with the stilbene derivative. The protective activity of ATP, together with the corresponding inhibitory effect of DIDS on both ATP transport and preprotein translocation (Figure 3), suggests that ATP transport

into microsomes is coupled to and essential for the translocation of preproteins across the membrane. This interpretation was strengthened by the observation that the irradiation of membranes in the presence of Bz_2-ATP also resulted in an inhibition of both ATP transport and preprotein translocation (Figure 5). From these results we can conclude that an ATP binding protein, sensitive to DIDS and Bz_2-ATP, is required for preprotein translocation.

Direct evidence linking ATP transport with translocation comes from our studies on selectively permeabilized membranes. In the presence of sub-solubilizing concentrations of octyl glucoside, ATP was able to diffuse from the medium into membrane vesicles, thereby by-passing the transport system. The fact that a modicum of translocation activity could be restored to membranes that were inactivated by 1 mM DIDS (Figure 7) shows that ATP is required in the lumen, and that the primary effect of DIDS on the translocation reaction is due to its inhibition of ATP transport.

Our kinetic analyses show that the transport of ATP into the lumen is not a rate-limiting step in the translocation process. In the case of both inhibitors examined, ATP uptake had to be reduced to a critical point before a rapid and linear decrease in translocation was observed (Figure 6). It is thus likely that ATP transport possesses a considerable excess capacity over translocation and can be reduced significantly (up to 80%) before an effect on translocation is seen. This is not an unexpected result as it is reasonable to assume that translocation is not the only reaction requiring luminal ATP. This observation also rules out the luminal ATP concentration as a factor regulating preprotein translocation.

In analogous situations, such as the import of proteins into mitochondria (Hwang and Schatz, 1989; Kang *et al.*, 1990; Scherer *et al.*, 1990) and chloroplasts (Pain and Blobel, 1987; Theg *et al.*, 1989), there is a requirement for ATP in the matrix and the stroma, respectively. These findings are in agreement with our results and support the notion that ATP is needed on the luminal side of the ER protein translocation apparatus. Recently, Clairmont *et al.* (1992) reported the existence of an enzyme-mediated ATP uptake system in mammalian rough ER-derived vesicles, although no relationship was established to the translocation process. For the translocation of precursor proteins into mammalian microsomes, an ATP requirement for post-translational (Klappa *et al.*, 1991) as well as for cotranslational modes of translocation (Klappa *et al.*, 1991; Zimmerman and Walter, 1991) was established. It would be most interesting to examine the connection between ATP uptake and translocation in a mammalian system in light of *in vitro* studies suggesting that luminal proteins are not needed for translocation (Bulleid and Freedman, 1988).

These data support and expand current models of protein translocation into yeast ER. Both genetic and biochemical analyses suggest that in post-translational translocation, the competence of preproteins is maintained by molecular chaperones such as the products of the *SSA1−4* genes (Deshaies *et al.*, 1988; Chirico *et al.*, 1988). The next observable step is an ATP-independent recognition of the preprotein by an ER-specific receptor (Sanz and Meyer, 1989), potentially the complex of Sec62/Sec63p (Deshaies and Schekman, 1991; Müsch *et al.*, 1992; Sanders *et al.*, 1992). Translocation across the lipid bilayer occurs via a pore whose composition includes Sec61p (Müsch *et al.*, 1992; Sanders *et al.*, 1992), and is driven by the folding of the precursor on the luminal side of the membrane, catalyzed by Kar2p (Sanders *et al.*, 1992). As ATP is required for Kar2p function, an important component of the translocation machinery is the transporter described here, whose function is to supply Kar2p with needed ATP. In assigning a protein or gene to this function, one of the six translocation specific *sec* mutants (Meyer, 1991; Green *et al.*, 1992) should be considered, although ATP binding motifs have not been found in the three *SEC* genes that have so far been sequenced (Sanders *et al.*, 1992). The ability to examine ATP transport activity in membranes from these mutants *in vitro*, coupled with the existence of radiolabeled forms of DIDS and ATP analogues, should hasten the identification of the membrane component(s) mediating ATP transport into the ER lumen.

Materials and methods

Materials

[α-^{32}P]ATP was purchased from New England Nuclear. Other nucleotides were obtained from United States Biochemical Corporation. DIDS, 3′-*O*-(4-benzoyl)benzoyl-ATP, 8-azido-ATP, carboxyatractyloside and trypsin were from Sigma. Proteinase K and creatine phosphokinase were purchased from Boehringer Mannheim.

ATP transport assay

Yeast microsomes were prepared according to Rothblatt and Meyer (1986a). For the determination of initial velocity of ATP uptake, aliquots of membranes, 50 μl (2 mg/ml) in membrane buffer (250 mM sucrose, 50 mM KOAc, 20 mM HEPES and 1 mM DTT, pH 7.4), were mixed rapidly with different concentrations of [α-^{32}P]ATP and incubated at 20°C. The transport assay was performed in the presence of 100 μM carboxyatractyloside to suppress uptake due to mitochondrial contaminants. The ATP uptake was stopped by rapid filtration of the samples through 60 mg of wet Dowex (1×8, chloride form, 100−200 mesh) in small glass columns. In this step, free ATP is tightly bound by the strong anion exchanger, whereas membrane vesicles are not retained. The column was washed once with 100 μl of water. Radioactivity in the combined eluate and wash was quantified by liquid scintillation counting. For calculating the amount of ATP transported specifically into the lumen of the vesicles, the minor portion of ATP associated with octyl glucoside-permeabilized vesicles was subtracted. Due to the light sensitivity of Bz_2-ATP, all competition studies with this analogue were carried out in the dark.

Modification of yeast microsomal membranes with DIDS

Yeast microsomes (25 μl, 2 mg/ml in membrane buffer) were incubated with DIDS (0.01−5 mM) for 15 min at 25°C. Subsequently, the microsomes were diluted 2.5-fold with membrane buffer and layered onto a 1 M sucrose cushion, 20 mM HEPES, pH 7.4. The samples were centrifuged in a Beckman TLA-100 rotor for 18 min at 300 000 *g*. The microsomal pellet was resuspended in membrane buffer to a concentration of 2 mg/ml.

Photoaffinity modification of yeast microsomal membranes with Bz_2-ATP

Aliquots of yeast microsomes (10 μl, 2 mg/ml in membrane buffer) were transferred into the wells of a microtiter plate placed on ice. Samples supplemented with different amounts of Bz_2-ATP were placed next to wells with control microsomes (without Bz_2-ATP). Subsequently the samples were exposed pairwise to UV-light (5 min), so that for every Bz_2-ATP concentration used, a control sample was irradiated identically. As light source a UV lamp, model B-100, 365 nm from Spectronics Corporation was used. Subsequently, the microsomes were diluted 2.5-fold with membrane buffer and layered onto a 1 M sucrose cushion, 20 mM HEPES, pH 7.4. The samples were centrifuged in a Beckman TLA-100 rotor for 18 min at 300 000 *g*. The microsomal pellet was resuspended in membrane buffer to a concentration of 2 mg/ml.

Construction of nonglycosylated prepro-α-factor

Site-directed mutagenesis (Kunkel *et al.*, 1987) was used to incorporate an *Eco*RV site (at the 3′ end of the globin cDNA) into the plasmid pSP64-XβM (Krieg and Melton, 1984). The three oligosaccharide-accepting asparagine

residues at positions 23, 57 and 67 in prepro-α-factor were mutated to glutamines simultaneously using two primers and site-directed mutagenesis (Kunkel *et al.*, 1987). The correct clone was verified by DNA sequencing. Then, PCR was used with the mutated prepro-α-factor cDNA to add an *Nco*I site to the 5′ end of prepro-α-factor. This construct was inserted into the modified pSP64-XβM vector, previously digested with *Eco*RV and *Nco*I to excise globin sequences, to form this plasmid pSP64αfΔCHO. The correct clone was isolated and linearized by *Eco*RI for *in vitro* transcription. For purification of radiochemical amounts of this prepro-α-factor construct using nickel ion affinity chromatography an oligonucleotide encoding six histidines was spliced into the coding region, 5′ to the naturally occurring stop codon (Bush *et al.*, 1991).

Purification of radiochemical amounts of prepro-α-factor(His)$_6$

Purification of radiochemical amounts of prepro-α-factor(His)$_6$ basically followed a modification of the procedure of Stüber *et al.* (1990) described previously (Bush *et al.*, 1991). A 2 ml translation mixture containing 35 μl of the mRNA construct described above and 800 μl yeast lysate was adjusted to the following concentrations: 42 mM HEPES, pH 7.4, 190 mM potassium acetate, 2.8 mM magnesium acetate, 20 mM creatine phosphate, 80 μg/ml creatine phosphokinase, 1 mM ATP, 0.1 mM GTP, 30 μM of each of 19 amino acids (minus methionine), 0.2 mg/ml yeast tRNA, 1500 units/ml of RNase inhibitor, 2 mCi of [^{35}S]L-methionine. After incubation for 1 h at 25°C the mixture was adjusted to 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris−HCl, pH 8.0 and was applied to a column (diameter 1 cm) filled with 1.2 ml Ni^{2+}-charged NTA resin which was packed and equilibrated in buffer A. For elution the column was washed sequentially with 3.5 ml of buffer B, C, D and E (buffer A: 8 M urea, 0.1 M NaH_2PO_4, pH 8.0; buffer B: 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris−HCl, pH 8.0; buffer C: same as B, pH 6.3; buffer D: same as B, pH 5.9, buffer E: same as B, pH 4.5). Each column fraction was adjusted to pH 7.0 with 2 M Tris−HCl, pH 9.5 and analyzed by SDS−PAGE and fluorography. The fractions containing radiochemically pure prepro-α-factor (usually the fraction eluted with buffer E) were combined and stored at −20°C.

Assays for translocation

In vitro transcription, translation and translocation of pSP64αfΔCHO were performed as described (Rothblatt and Meyer, 1986a,b). Translocation of purified prepro-α-factor(His)$_6$ by dilution out of urea was performed as described previously (Sanz and Meyer, 1988). Quantification of translocation products was carried out on a computer-assisted Geiger densitometer equipped with AMBIS QuantProbe Software.

Proteinase K treatments of translation and translocation products

Proteinase K digestion was performed at 0°C for 60 min at a final concentration of 0.5 mg/ml. Proteolysis was stopped by the addition of PMSF (20 mg/ml in isopropanol) to a final concentration of 1.5 mg/ml. After incubation for 5 min at 0°C, an equal amount of SDS−PAGE sample buffer was added, and the samples were heated to 95°C for 3 min.

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