Biochimica et Biophysica Acta, 1150 (1993) 89–97

BBAMEM 76033

Puromycin inhibits protein import into mitochondria by interfering with an intramitochondrial ATP-dependent reaction

Jeannie Price and Keith Verner

Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, PA (USA)

(Received 20 April 1993)

Key words: Mitochondrial biogenesis; Puromycin; Protein transport; Biogenesis; ATP; Precursor protein; (Mitochondrion)

We have performed experiments which demonstrate that puromycin inhibits the import of proteins into mitochondria in in vitro reactions containing mitochondria isolated from the yeast *Saccharomyces cerevisiae* and precursor proteins synthesized in a nuclease-treated rabbit reticulocyte lysate. Puromycin inhibited the import of several precursor proteins including; a fusion protein consisting of the first 22 N-terminal residues of yeast cytochrome oxidase subunit IV fused to mouse dihydrofolate reductase, both a destabilized and truncated form of this same fusion protein, the β-subunit of the yeast mitochondrial F_1-ATPase and yeast alcohol dehydrogenase III. The insertion of the yeast outer mitochondrial protein porin was not inhibited by puromycin. Puromycin-induced import inhibition could be overcome by adding additional ATP to the import reactions. However, if access of ATP to the mitochondrial matrix was prevented by blocking the adenine nucleotide translocase with carboxyatractyloside, ATP addition was unable to overcome the inhibitory effect of puromycin on protein import. Collectively, these results demonstrate that puromycin inhibits protein import into mitochondria by interfering with an ATP-dependent step in the import process and that the ATP-dependent component in the reaction is located inside the inner mitochondrial membrane. In addition to supporting the view that ATP is required in the matrix for efficient protein import, these results may provide a useful tool for identifying the ATP-binding components of the import apparatus.

Introduction

The vast majority of mitochondrial proteins are encoded by nuclear DNA and synthesized on cytosolic ribosomes. Consequently, many of these proteins must be transported across both the mitochondrial membranes before they arrive at their final destinations, while others are inserted directly into the outer mitochondrial membrane. The mechanism by which specific proteins are targeted to mitochondria and the mechanism by which they are subsequently imported into the organelle has been the subject of widespread interest for over a decade [1–4].

Import of proteins into the mitochondrial matrix requires the hydrolysis of ATP in addition to an energized mitochondrial inner membrane [5–7]. However, the function of ATP in these reactions is not entirely clear. Certainly, a clue to the function of ATP in the import process may come from the precise location at which it is required. For example, the reported involvement of heat shock-like proteins inside the mitochondrial matrix suggests a possible site for ATP involvement in the import process. However, there are conflicting reports as to whether the nucleotide is needed on the inside or outside of the mitochondrial inner membrane [5,6,8,9]. In addition, there may well be multiple ATP-dependent reactions involved in the import process.

In a recent study, we found that the addition of puromycin to a posttranslational import reaction, consisting of isolated yeast mitochondria and a precursor protein synthesized in a nuclease-treated reticulocyte lysate, had an inhibitory effect on import [10]. In this report we present evidence that the inhibitory effect of puromycin on protein import is due to its interference with an ATP-dependent step in the import reaction that takes place inside the mitochondrial inner membrane.

Materials and Methods

Yeast strains and plasmids. Mitochondria were isolated from *Saccharomyces cerevisiae* strain D273-10B (ATCC 25657). The plasmids used for in vitro transcription of COXIV-DHFR fusion genes consisting of the first 22 and 12 amino-terminal amino acid residues of yeast cytochrome oxidase subunit IV fused to mouse

Correspondence to: K. Verner, Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, PA 17033, USA.

dihydrofolate reductase were pDS5/2-1-pCOXIV(1–22)DHFR and pDS5/2-1-pCOXIV(1–12)DHFR, respectively [11,12]. Plasmid pdCOXIV(1–22)DHFR was constructed by exchanging restriction fragments between plasmid pDS5/2-1-pCOXIV(1–22)DHFR and a similar plasmid provided by Dr. D. Vestweber who introduced cysteines at DHFR positions 157 and 189 by site-directed mutagenesis as described [13]. Plasmid pGR207, encoding the precursor form of the β-subunit of the yeast mitochondrial F_1-ATPase under the control of the T5 promoter, was a gift from Dr. Graeme Reid of the University of Edinburgh. Plasmid pT7Por4 [10] was used for transcription of the yeast porin gene and plasmid pDS5/2-pADH was used for transcription of the yeast alcohol dehydrogenase isozyme III precursor [14].

In vitro transcription and translation. Capped mRNA was synthesized from all plasmids except plasmid pT7Por4 essentially as described [12]. Plasmid pT7Por4 was linerized by digestion with *Hin*dIII and transcribed in the presence of the cap analog *P'*-5-(7-methyl)guanosine-P^3-5'-guanosine triphosphate as described [10]. Published procedures were used for the in vitro translation of capped transcripts in a nuclease-treated rabbit reticulocyte lysate [15].

Posttranslational in vitro import reactions. Yeast mitochondria were isolated [16] from yeast strain D273-10B.. Mitochondria were incubated with radiolabeled proteins essentially as described [17]. Incubations contained 200 μg of mitochondrial protein and 10 μl of radiolabeled products from in vitro translation reactions. After incubation for 30 min at 30°C, mitochondria were reisolated by centrifugation through a sucrose cushion. For digestion of externally located radiolabeled polypeptides following the import reaction, the mitochondria were incubated for 15 min at 0°C with 250 μg/ml proteinase K. The proteinase was inhibited with 1 mM phenylmethylsulfonyl fluoride (PMSF) and the mitochondria reisolated as above.

Miscellaneous. Published procedures were used for SDS-polyacrylamide gel electrophoresis and fluorography [17]. Protein was measured by the Bio-Rad protein assay according to the manufacturer's instructions. Fluorograms were scanned using a 'Soft Laser' scanning densitometer from LKB Instruments. Membrane potential measurements were performed in the same buffer used for import reactions using the dye safranine O (Aldrich) essentially as described [18]. Oxygen consumption was measured polarographically.

Results

Puromycin inhibits the posttranslational import of proteins into isolated yeast mitochondria

Previous results suggested that the addition of puromycin to an in vitro mitochondrial import reaction containing isolated yeast mitochondria and a precursor protein synthesized in a nuclease pretreated rabbit reticulocyte lysate partially inhibited the import of the precursor protein while the addition of cycloheximide had no inhibitory effect [10]. Consequently, inhibition of protein synthesis alone appears to be unable to explain the mechanism of puromycin import inhibition.

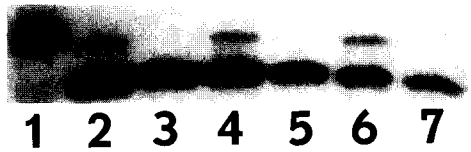

Fig. 1. Effect of puromycin on import of COXIV(1–22)DHFR into isolated yeast mitochondria. The COXIV(1–22)DHFR fusion protein was synthesized in a nuclease-treated rabbit reticulocyte lysate for 30 min at 30°C. Aliquots of the reaction were then added to 0.2 mg freshly isolated yeast mitochondria in a final volume of 200 μl (see Materials and Methods) and incubated for 30 min at 30°C either with no additions (lanes 2 and 3), with the addition of 2 mM puromycin (lanes 4 and 5) or with 4 mM puromycin (lanes 6 and 7). The mitochondria were then reisolated and analyzed by SDS-12% polyacrylamide gel electrophoresis and fluorography. Where indicated, mitochondria were first treated with 250 μg/ml proteinase K for 15 min at 0°C and then with 1 mM PMSF before reisolation. Lanes: (1), 20% of the labeled COXIV(1–22)DHFR which was added to the mitochondrial samples shown in lanes 2–7; (2), after incubation with mitochondria; (3), mitochondria treated with proteinase K; (4), after incubation with mitochondria and 2 mM puromycin; (5), mitochondria and 2 mM puromycin, treated with proteinase K; (6), after incubation with mitochondria and 4 mM puromycin; (7), mitochondria and 4 mM puromycin, treated with proteinase K.

In order to examine the effect of puromycin on protein import in greater detail, we added the drug to in vitro import reactions containing isolated yeast mitochondria (Fig. 1). The precursor protein used in this experiment was a fusion protein consisting of the N-terminal mitochondrial targeting signal of yeast cytochrome oxidase subunit IV fused to the mouse cytosolic enzyme dihydrofolate reductase (the COXIV(1–22)DHFR fusion protein). This precursor protein has been shown to be efficiently imported into yeast mitochondria both in vitro and in vivo [11]. As can be seen, in the absence of puromycin, the COXIV(1–22)DHFR fusion protein (lane 1) was reisolated with the mitochondria after a 30 min incubation at 30°C and cleaved to a lower, mature form (lane 2). This cleavage is due to the specific removal of the mitochondrial targeting signal by a chelator-sensitive, matrix-located processing proteinase. In addition, a small amount of the precursor form of the fusion protein, which is bound to but not imported into the mitochondria, is also present (lane 2). If the mitochondria were treated with proteinase K before reisolation, all of the surface bound precursor was degraded and only the imported, mature form of the protein was protected (lane 3). Densitometric scanning of the fluorogram shown in Fig. 1 demonstrated that 22% of the

precursor protein added to the mitochondria was imported in the absence of puromycin. On the other hand, when puromycin was added to a final concentration of 2 mM, import was reduced to 18% of the precursor added (approx. 18% inhibition, lane 5). The amount of import was further reduced to 9% of the precursor added when the puromycin concentration was increased to 4 mM (approx. 60% inhibition, lane 7). Puromycin had no effect on the amount of the higher molecular weight precursor form reisolated with the mitochondria (compare lanes 2, 4 and 6), suggesting that the drug neither inhibits the binding of precursor to the mitochondrial membrane nor induces precursor insolubility. From these results, it is clear that puromycin can inhibit the import of the precursor protein into isolated mitochondria and that this inhibition is independent of the synthesis of the precursor protein itself.

In experiments to further characterize the puromycin effect, we found that the extent of import inhibition induced by the drug varied considerably from one experiment to another. This was particularly true when mitochondria were incubated at 0°C for extended periods, in which case the sensitivity to puromycin increased. In the experiment presented in Table I, the inhibitory effect of 2 mM puromycin on the import of COXIV(1–22)DHFR into freshly isolated mitochondria and into the same mitochondrial preparation incubated on ice for 20 and 44 h prior to the import reaction is shown. As can be seen, 2 mM puromycin inhibited import by only about 13% when freshly prepared mitochondria were tested. However, when the same preparation of mitochondria were incubated for an additional 20 h on ice and then tested for import, 2 mM puromycin inhibited import by 77%. An additional 24 h on ice resulted in complete inhibition of import by 2 mM puromycin. On the other hand, the same mitochondria, regardless of preincubation time, imported the precursor protein in the absence of puromycin with about the same efficiency as when freshly prepared. Therefore, some other property of the preincubated mitochondria, other than their overall ability to import proteins, must be related to the puromycin effect.

TABLE I

Effect of mitochondrial preincubation time on puromycin inhibition of COXIV(1–22)DHFR import

The COXIV(1–22)DHFR fusion protein was synthesized in a nuclease-treated rabbit reticulocyte lysate for 30 min at 30°C and then incubated with 0.2 mg mitochondria that were either freshly isolated or incubated at 0°C for 20 h or 44 h. Import was performed either without additions or with the addition of 2 mM puromycin. All samples were treated with 250 μg/ml proteinase K for 15 min at 0°C and then with 1 mM PMSF before reisolating the mitochondria by centrifugation. Following SDS-12% polyacrylamide gel electrophoresis and fluorography, the percent import in each reaction was determined by densitometric scanning of the fluorogram.

Time (h)	% Import		% inhibition
	− puromycin	+ puromycin	
0	15	13	13
20	22	5	77
44	13	0	100

TABLE II

Effect of puromycin on the import of various mitochondrial precursor proteins

Various mitochondrial precursor proteins were synthesized in a nuclease-treated rabbit reticulocyte lysate for 30 min at 30°C. Aliquots of the translation reactions (10 μl) were then added to 0.2 mg isolated mitochondria (preincubated at 0°C for 20 h), either in the presence or absence of 2 mM puromycin. After 30 min at 30°C, mitochondrial samples were treated with 250 μg/ml proteinase K for 15 min at 0°C and then with 1 mM PMSF before reisolation and analysis by SDS-polyacrylamide gel electrophoresis and fluorography. The percent import and import inhibition by puromycin were determined by densitometric scanning of the fluorograms.

Precursor protein	% Import		% inhibition
	− puromycin	+ puromycin	
COXIV(1–22)DHFR	20	3	85
$F_1\beta$	20	2	90
ADHIII	15	3	80
dCOXIV(1–22)DHFR	31	4	87
COXIV(1–12)DHFR	17	3	82

In order to determine if puromycin inhibited the import of other precursor proteins, we performed a series of import reactions in which various precursor proteins were synthesized in a nuclease-treated rabbit reticulocyte lysate and then added to mitochondria that had been preincubated for 20 h at 0°C (Table II). In this experiment, the import of COXIV(1–22)DHFR was inhibited by about 85% by 2 mM puromycin. The precursors of two authentic yeast mitochondrial proteins, the β-subunit of the mitochondrial F_1-ATPase ($F_1\beta$) and alcohol dehydrogenase III (ADHIII) were inhibited by 90% and 80%, respectively, indicating that puromycin-induced import inhibition is not limited to artificial fusion precursor proteins. Since the unfolding of precursor proteins has been suggested to be a rate limiting step in the import process, we tested the effect of puromycin treatment on the import of a 'destabilized' form of COXIV(1–22)DHFR. This precursor protein (dCOXIV(1–22)DHFR) was generated by the introduction, by site-directed mutagenesis, of cysteine residues into the DHFR moiety of COXIV(1–22)DHFR and exhibits many properties of an unfolded precursor protein [13]. However, dCOXIV(1–22)DHFR was just as suseptible to puromycin inhibition as COXIV(1–22)DHFR (Table II). Finally, to determine if puromycin inhibits import into isolated mitochondria by interfering with the cleavage of the presequence from precursor proteins, we analyzed the effect of

puromycin treatment on COXIV(1–12)DHFR. This precursor protein is identical to COXIV(1–22)DHFR except that it consists of only the first 12 amino acid residues of the cytochrome oxidase subunit IV presequence fused to mouse DHFR and lacks the matrix proteinase cleavage site [11]. As can be seen, the import of this non-cleavable precursor protein was inhibited to approximately the same extent as COXIV(1–22)DHFR, which contains the matrix proteinase cleavage site.

Puromycin-induced import inhibition can be overcome by the addition of ATP

In vitro import is known to require ATP in addition to an energized mitochondrial inner membrane [5–7]. Since puromycin shares significant structural similarities with ATP, we considered that it may compete with ATP in the import process. Further, the increased puromycin sensitivity of mitochondrial samples preincubated for extended periods on ice (Table I) could be related to decreased concentrations of ATP in these preparations. If this were the case, increasing the total ATP concentration in the import reaction might overcome the inhibitory effect of puromycin. We tested this possibility in the experiment shown in Fig. 2. For these experiments we used mitochondria that were preincubated for 20 h on ice; a pretreatment that reproducibly results in high import efficiency as well as maximal import inhibition at reasonable puromycin concentrations (see Table I). The import of both the COXIV(1–22)DHFR fusion protein (lower panel) and the precursor of the authentic yeast mitochondrial protein alcohol dehydrogenase III (pADHIII, upper panel) were analyzed. In the absence of puromycin, approx. 20% of the precursors added to the import reactions were reisolated with the mitochondria (lanes 2) and were resistant to proteinase treatment (lanes 3). The addition of 2 mM puromycin drastically inhibited the import of both precursor proteins (lanes 5). However, puromycin-induced import inhibition could be largely overcome if ATP was added to the reactions (lanes 7). In the experiment shown in Fig. 2, ATP was added to a final concentration of 10 mM. However, restoration of COXIV(1–22)DHFR import in the presence of 2 mM puromycin could be achieved with lower ATP concentrations (Fig. 3).

While it is clear that the addition of ATP can overcome puromycin-induced import inhibition, we wished to determine if ATP hydrolysis was required. To do so, we used the non-hydrolysable ATP analog 5′-adenylyl imidodiphosphate (AMP · PNP). In the experiment shown in Table III, the addition of 2 mM puromycin inhibited the import of COXIV(1–22)DHFR by 77%. When ATP was added along with puromycin, import was restored to control levels. However, when AMP · PNP was added in place of ATP together with puromycin, no restoration of import was observed. Consequently, this non-hydrolysable ATP analog is unable to substitute for ATP in overcoming puromycin-induced import inhibition. Finally, we added both ATP and AMP · PNP along with puromycin to determine the effect of the non-hydrolysable analog on the ability of ATP to overcome puromycin-induced import inhibition. As can be seen, while some restora-

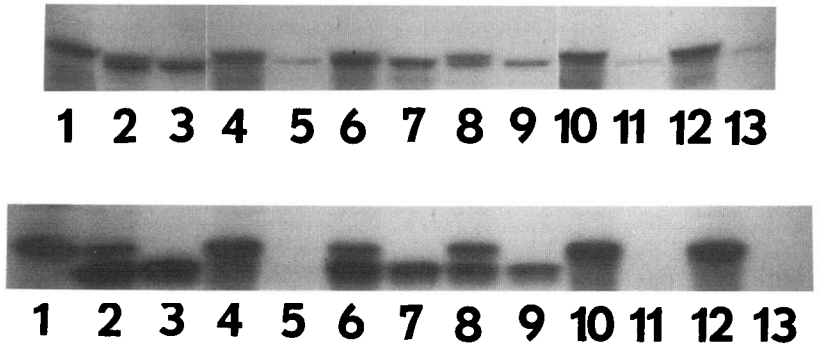

Fig. 2. Effect of puromycin, ATP and carboxyatractyloside on the import of COXIV(1–22)DHFR and pADHIII into isolated yeast mitochondria. The COXIV(1–22)DHFR fusion protein (lane 1, lower panel) and pADHIII (lane 1, upper panel) were synthesized in a nuclease-treated reticulocyte lysate for 30 min at 30°C. Aliquots (10 μl) were then added to 0.2 mg isolated mitochondria that were preincubated at 0°C for 20 h and then incubated with the additions described below for 30 min at 30°C. The mitochondria were then reisolated by centrifugation and analyzed by SDS-12% polyacrylamide gel electrophoresis and fluorography. Where indicated, mitochondria were first treated with 250 μg/ml proteinase K for 15 min at 0°C and then with 1 mM PMSF before reisolation. Lanes: (1), 20% of either labeled COXIV(1–22)DHFR (lower panel) or pADHIII (upper panel) added to the mitochondrial samples shown in lanes 2–13; (2), after incubation with mitochondria; (3), mitochondria, treated with proteinase K; (4), after incubation with mitochondria and 2 mM puromycin; (5), mitochondria and 2 mM puromycin, treated with proteinase K; (6), after incubation with mitochondria, 2 mM puromycin and 10 mM ATP; (7), mitochondria, 2 mM puromycin and 10 mM ATP, treated with proteinase K; (8), after incubation with mitochondria and 150 μg/ml carboxyatractyloside; (9), mitochondria, 150 μg/ml carboxyatractyloside, treated with proteinase K; (10), after incubation with mitochondria, 150 μg/ml carboxyatractyloside and 2 mM puromycin; (11), mitochondria, 150 μg/ml carboxyatractyloside, 2 mM puromycin, treated with proteinase K; (12), after incubation with mitochondria, 150 μg/ml carboxyatractyloside, 2 mM puromycin and 10 mM ATP; (13), mitochondria, 150 μg/ml carboxyatractyloside, 2 mM puromycin, 10 mM ATP, treated with proteinase K.

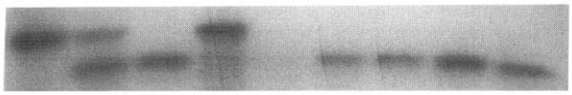

Fig. 3. Effect of 2 mM puromycin on COXIV(1–22)DHFR import into isolated yeast mitochondria in the presence of increasing ATP concentrations. The COXIV(1–22)DHFR fusion protein was synthesized in a nuclease-treated rabbit reticulocyte for 30 min at 30°C and then aliquots (10 μl) were added to isolated yeast mitochondria preincubated at 0°C for 20 h as in Fig. 2. Mitochondria were then reisolated by centrifugation and analyzed by SDS-12% polyacrylamide gel electrophoresis and fluorography. Where indicated, mitochondria where first treated with 250 μg/ml proteinase K for 15 min at 0°C and then with 1 mM PMSF before reisolation. Lanes: (1), 20% of the labeled COXIV(1–22)DHFR added to the mitochondrial samples shown in lanes 2–9; (2), after incubation with mitochondria; (3), mitochondria, treated with proteinase K; (4), after incubation with mitochondria and 2 mM puromycin; (5), mitochondria, 2 mM puromycin, treated with proteinase K; (6), mitochondria, 2 mM puromycin, 1.25 mM ATP, treated with proteinase K; (7), mitochondria, 2 mM puromycin, 2.5 mM ATP, treated with proteinase K; (8), mitochondria, 2 mM puromycin, 5 mM ATP, treated with proteinase K; (9), mitochondria, 2 mM puromycin, 10 mM ATP, treated with proteinase K.

tion is observed, AMP · PNP reduces the extent to which ATP is capable of overcoming puromycin-induced import inhibition. This finding suggests that AMP · PNP may compete with ATP for the same binding site.

ATP can not overcome puromycin-induced import inhibition if its access to the mitochondrial matrix is blocked by carboxyatractyloside

The observation that ATP addition overcomes puromycin-induced import inhibition suggests that puromycin may inhibit protein import into mitochondria by interacting with an ATP-dependent component of the import machinery. We therefore wished to determine the location of such a component. In order to do so, we included carboxyatractyloside in the import reactions (Fig. 2, lanes 8–13). Carboxyatractyloside inhibits the mitochondrial adenine nucleotide translocator and prevents externally added ATP from entering mitochondria [19]. As can be seen, the addition of carboxyatractyloside alone has some inhibitory effect on protein import under these conditions (compare lanes 9 to lanes 3). Nonetheless, the addition of 2 mM puromycin dramatically inhibited the import of both precursor proteins (lanes 11). However, when the translocation of ATP into the matrix was blocked by carboxyatractyloside, the addition of ATP to the reaction did not overcome the puromycin-induced import inhibition (lanes 13). In these experiments, the addition of ATP alone had no effect on the import of precursor proteins into mitochondria either in the presence or absence of carboxyatractyloside. While preincubated mitochondria were used in the experiment shown in Fig. 2, identical results were obtained with freshly prepared mitochondria, although higher puromycin concentrations were required to induce the same degree of import inhibition (not shown). Collectively, these results suggest that puromycin interferes with an ATP-dependent step in the import process and that the ATP-dependent component in the reaction is located inside the mitochondrial inner membrane.

TABLE III

Effect of ATP and AMP·PNP on puromycin-induced import inhibition of COXIV(1–22)DHFR

The COXIV(1–22)DHFR fusion protein was synthesized in a nuclease-treated rabbit reticulocyte lysate for 30 min at 30°C and then incubated with 0.2 mg mitochondria that were preincubated at 0°C for 20 h. Import was performed either without additions or with the various additions indicated. All samples were treated with 250 μg/ml proteinase K for 15 min at 0°C and then with 1 mM PMSF before reisolating the mitochondria by centrifugation. Following SDS-12% polyacrylamide gel electrophoresis and fluorography, the percent import in each reaction was determined by densitometric scanning of the fluorogram. The results are expressed as the percent of a control reaction (None) that received no treatment. Import efficiency for the control reaction was 22% in this experiment.

Treatment	% of control
None	100
2 mM puromycin	23
2 mM puromycin + 10 mM ATP	102
2 mM puromycin + 10 mM AMP·PNP	22
2 mM puromycin + 10 mM ATP + 10 mM AMP·PNP	50

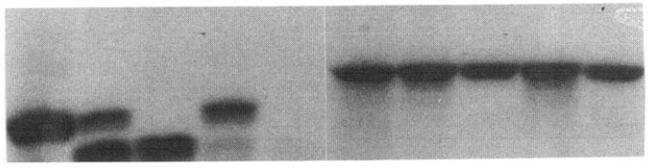

Fig. 4. Effect of puromycin on the import of COXIV(1–22)DHFR and yeast porin. The COXIV(1–22)DHFR fusion protein and yeast porin were synthesized in a nuclease-treated rabbit reticulocyte lysate for 30 min at 30°C. Aliquots (10 μl) of the translation reactions were then added to mitochondria preincubated at 0°C for 20 h as in Fig. 2, either with no additions (lanes 2, 3, 7 and 8) or with the addition of 2 mM puromycin (lanes 4, 5, 9 and 10). The mitochondria were then reisolated by centrifugation and analyzed by SDS-12% polyacrylamide gel electrophoresis and fluorography. Where indicated, mitochondria were first treated with 250 μg/ml proteinase K for 15 min at 0°C and then 1 mM PMSF before reisolation. Lanes: (1), 20% of the labeled COXIV(1–22)DHFR added to the mitochondrial samples shown in lanes 2–5; (2), after incubation with mitochondria, (3), mitochondria, treated with proteinase K; (4), after incubation with mitochondria and 2 mM puromycin; (5), mitochondria, 2 mM puromycin, treated with proteinase K; (6), 20% of the labeled yeast porin added to the mitochondrial samples shown in lanes 7–10; (7), after incubation with mitochondria; (8), mitochondria, treated with proteinase K; (9), after incubation with mitochondria and 2 mM puromycin; (10), mitochondria, 2 mM puromycin, treated with proteinase K.

It has been demonstrated that while ATP is needed for the insertion of the yeast outer mitochondrial membrane protein porin, the nucleotide is required only at a location outside the mitochondrial inner membrane [8]. Therefore, if puromycin inhibits precursor import into the mitochondrial matrix by interfering with an ATP-dependent reaction inside the inner membrane, addition of the drug should have no inhibitory effect on the in vitro insertion of porin. This proved to be the case (Fig. 4). Both the COXIV(1–22)DHFR fusion protein (lane 1) and yeast porin (lane 6) were synthesized in a rabbit reticulocyte lysate and then added to mitochondria preincubated for 20 h on ice. In the absence of puromycin, approx. 20% of the COXIV(1–22)DHFR was imported (lane 3). Similarly, about 20% of the radiolabeled porin was inserted into the mitochondrial outer membrane (lane 8). In the presence of 2 mM puromycin, the import of COXIV(1–22)DHFR was drastically inhibited (lane 5), while the insertion of porin was unaffected (lane 10). Therefore, puromycin does not inhibit the insertion of yeast porin into the outer mitochondrial membrane.

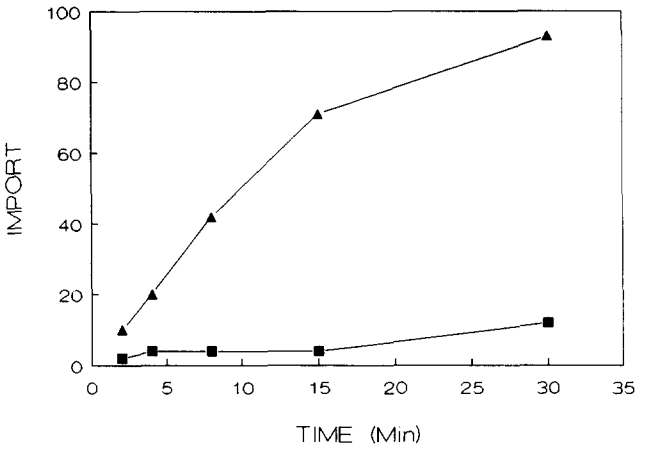

Fig. 5. Effect of 2 mM puromycin on the time-course of COXIV(1–22)DHFR import. The COXIV(1–22)DHFR fusion protein was synthesized in a nuclease-treated rabbit reticulocyte lysate as in Fig. 2. Aliquotes (10 μl) were added to mitochondria that were preincubated for 20 h at 0°C. Incubations were at 30°C either with or without the addition of 2 mM puromycin for the times indicated. After incubation, mitochondria were treated with 250 μg/ml proteinase K for 15 min at 0°C and then with 1 mM PMSF. Mitochondria were then reisolated by centrifugation and analyzed by SDS-12% polyacrylamide gel electrophoresis and fluorography. The results of densitometric scanning of the fluorogram are presented. ▲, No additions; ■, 2 mM puromycin added. The *y*-axis is in arbitrary units.

Puromycin-induced import inhibition is reversible and is not related to an effect on the mitochondrial membrane potential

The import of proteins into mitochondria is dependent upon a potential across the inner mitochondrial membrane [1–3]. It was therefore essential to rule out the possibility that puromycin inhibits proteins from entering mitochondria by collapsing the membrane potential. We tested the effect of the addition of 2 mM puromycin on the membrane potential of mitochondria that were preincubated for 20 h on ice. Potential measurements were performed in a buffer identical to that used in the import reactions (see Materials and Methods). We found that the addition of puromycin at a concentration that inhibits protein import has no effect on the membrane potential. In addition, when mitochondria were analyzed using an oxygen measurement system, puromycin at twice the concentration required for import inhibition had no effect on either the respiratory rate or respiratory control ratio (not shown).

It could be argued that while puromycin had no apparent effect on mitochondrial respiration or membrane potential in the above experiments, the damage to mitochondria caused by the drug may only be manifested with time during the course of a 30 min import reaction; that is, import may be relatively unaffected early in an import reaction but the puromycin-treated mitochondria may loose their ability to import proteins at some later point in the incubation. To test this possibility, we followed the time-course of COXIV(1–22)DHFR import into mitochondria with and without the addition of 2 mM puromycin (Fig. 5). As can be seen, the inhibitory effect of puromycin is clearly evident at the earliest time points taken and well within the time frame of our membrane potential and respiration measurements. Consequently, it does not appear that puromycin-induced import inhibition is caused by mitochondrial de-energization.

While the experiments described above demonstrate that puromycin does not significantly affect mitochondrial energetics, it is nonetheless possible that it damages mitochondria by some other, undefined mechanism. If this were the case, preincubation of mitochondria with puromycin might be expected to produce the same effect as adding the drug directly to the import reaction. We examined this possibility by preincubating mitochondria for 10 min with 5 mM puromycin. As a control, an aliquot of the mitochondrial preparation was treated identically except that no puromycin was included in the preincubation. Mitochondria were then reisolated to remove unbound drug and immediately analyzed for import of COXIV(1–22)DHFR (Fig. 6). As can be seen, the inclusion of 5 mM puromycin in the mitochondrial pretreatment had little effect on the ability of the mitochondria to import and process the precursor protein (compare lanes 3 and 7). On the other hand, the addition of only 2 mM puromycin directly to the import reactions drastically inhibited import (lanes 5 and 9). It is therefore apparent that puromycin does not irreversibly damage iso-

Fig. 6. Effect of preincubating mitochondria with puromycin on the import of COXIV(1–22)DHFR. Mitochondria that were incubated for 20 h at 0°C were further incubated for 10 min either with or without the addition of 5 mM puromycin. The mitochondria were then reisolated by centrifugation and used in import reactions. The COXIV(1–22)DHFR fusion protein was synthesized in a rabbit reticulocyte lysate for 30 min at 30°C. Aliquots (10 μl) were then added to the control mitochondrial samples (lanes 2–5) and the puromycin-pretreated mitochondrial samples (lanes 6–9). After incubation for 30 min at 30°C, the mitochondria were reisolated by centrifugation and analyzed by SDS-12% polyacrylamide gel electrophoresis and fluorography. Where indicated, mitochondria were treated with 250 μg/ml proteinase K for 15 min at 0°C and then 1 mM PMSF before reisolation. Lanes: (1), 20% of the labeled COXIV(1–22)DHFR added to the mitochondrial samples shown in lanes 2–9; (2), after incubation with control mitochondria; (3), control mitochondria, treated with proteinase K; (4), after incubation with control mitochondria and 2 mM puromycin; (5), control mitochondria, 2 mM puromycin, treated with proteinase K; (6), after incubation with puromycin-pretreated mitochondria; (7), puromycin-pretreated mitochondria, treated with proteinase K; (8), after incubation with puromycin-pretreated mitochondria and 2 mM puromycin; (9), puromycin-pretreated mitochondria, 2 mM puromycin, treated with proteinase K.

lated mitochondria in terms of their ability to import proteins.

Finally, to exclude the possibility that puromycin-induced import inhibition is actually due to increased degradation of the imported precursor protein rather than import inhibition per se, we performed the experiment shown in Fig. 7. The COXIV(1–22)DHFR fusion protein (lane 1) was added to mitochondria and import was performed for 30 min at 30°C. In this experiment, approx. 20% of the fusion protein was imported (lane 3). If the mitochondria were deenergized by including valinomycin in the reaction, import was completely inhibited (lane 5). As before, the addition of 2 mM puromycin resulted in a dramatic decrease in the amount of imported COXIV(1–22)DHFR (lane 7). Next, we added the fusion protein to mitochondria in the absence of either valinomycin or puromycin and incubated for 30 min at 30°C. We then added valinomycin to inhibit further import and incubated for an additional 30 min at 30°C either in the absence (lane 8) or presence (lane 9) of 2 mM puromycin before treating with proteinase K and reisolating the mitochondria. As can clearly be seen, puromycin had absolutely no effect on the stability of imported COXIV(1–22) DHFR.

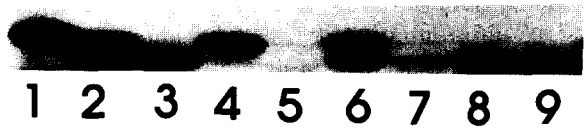

Fig. 7. Effect of puromycin on the stability of imported COXIV(1–22)DHFR. The COXIV(1–22)DHFR fusion protein was synthesized in a nuclease-treated rabbit reticulocyte lysate for 30 min at 30°C. Aliquots (10 μl) of the translation reactions were then added to mitochondria that were preincubated at 0°C for 20 h as in Fig. 2. The mitochondria were then reisolated by centrifugation and analyzed by SDS-12% polyacrylamide gel electrophoresis and fluorography. Where indicated, mitochondria were first treated with 250 μg/ml proteinase K for 15 min at 0°C and then 1 mM PMSF before reisolation. Lanes: (1), 20% of the labeled COXIV(1–22)DHFR added to the mitochondrial samples shown in lanes 2–9; (2), after incubation with mitochondria; (3), mitochondria, treated with proteinase K; (4), after incubation with mitochondria and 1 μg/ml valinomycin; (5), mitochondria and 1 μg/ml valinomycin, treated with proteinase K; (6), after incubation with mitochondria and 2 mM puromycin; (7), mitochondria and 2 mM puromycin, treated with proteinase K; (8), incubation with mitochondria for 30 min at 30°C, 1 μg/ml valinomycin was then added and incubation continued for an additional 30 min, treated with proteinase K; (9), incubation with mitochondria for 30 min at 30°C, 1 μg/ml valinomycin and 2 mM puromycin were then added and incubation continued for an additional 30 min, treated with proteinase K.

Discussion

In this report, we have demonstrated that puromycin can inhibit the import of precursor proteins into mitochondria in an in vitro system consisting of isolated yeast mitochondria and precursor proteins synthesized in a nuclease-treated reticulocyte lysate. As protein synthesis is unable to occur under these conditions, it is clear that the inhibitory effect of puromycin on the import process is not directly related to precursor synthesis in this heterologous system. Also, we have previously shown that cycloheximide does not inhibit precursor import under the same conditions as does puromycin [10].

Why does puromycin inhibit protein import into mitochondria? The import of proteins is known to be dependent upon an energized mitochondrial membrane. Consequently, a trivial explanation for puromycin's inhibitory effect on import could be that it collapses the membrane potential. However, puromycin had no detectable effect on the mitochondrial membrane potential as demonstrated by the use of the potential-sensitive dye safranine (Fig. 5). Neither was there an effect of puromycin addition on respiration rates or respiratory control ratios, suggesting that puromycin does not inhibit the mitochondrial F_1-ATPase (which would result in decreased respiration), increase intramitochondrial ADP concentrations (which would result in increased respiration) nor inhibit the adenine nucleotide translocator (which would make the isolated mitochondria insensitive to ADP addition). The lack of a puromycin effect in these experiments suggests that the drug does not directly affect mitochondrial energetics. In addition, we previously demonstrated that inhibition of mitochondrial protein

synthesis by chloramphenicol does not inhibit in vitro import [10]. It is therefore unlikely that puromycin inhibits protein import into mitochondria indirectly by interfering with intramitochondrial translation. These results are consistent with an earlier study with isolated rat liver mitochondria, which demonstrated that puromycin had no effect on either respiratory control or O_2 consumption, even though mitochondrial protein synthesis was inhibited [20].

The observation that ATP can overcome the inhibitory effect of puromycin on the import process suggests that the drug interferes with an ATP-dependent reaction. Further, the finding that the non-hydrolysable ATP analog, AMP · PNP, can not replace ATP to overcome the inhibitory effect of puromycin suggests that this reaction involves ATP hydrolysis. While ATP has been demonstrated to be essential for the import of proteins into mitochondria, the precise function of the nucleotide in the import reaction is not entirely clear. It has been suggested, for example, that ATP may be required for the unfolding of proteins by cytosolic factors, perhaps heat shock-like proteins, in order to generate or maintain precursors in import competent conformations [21–26]. However, several of our findings suggest that puromycin does not inhibit this type of reaction. First, dramatically different degrees of import inhibition are observed at the same puromycin concentration when mitochondria are preincubated for various periods of time, even though the lysates in such experiments are identical (Table I). By the same argument, it is unlikely that puromycin directly interacts with mitochondrial precursor proteins and inhibits their import. Second, porin requires ATP for insertion into the outer mitochondrial membrane and evidence exists suggesting that ATP may be involved in the generation of an unfolded porin conformation, perhaps relying on cytosolic factors [27]. The inability of puromycin to inhibit porin insertion indicates that the drug does not interfere with ATP in this process. Finally, based on experiments using carboxyatractyloside to block added ATP from reaching the matrix (Fig. 2), it is clear that the puromycin-inhibitable, ATP-dependent reaction occurs inside the mitochondrial inner membrane. In contrast, cytosolic factors, either soluble components of the reticulocyte lysate or endogenous yeast cytosolic factors bound to the isolated organelle, would be located on the outside of the outer membrane.

In a recent study, puromycin was shown to affect the membrane conductance of a system in which microsomal membranes were incorporated into lipid bilayers [28]. It was suggested that the effect of puromycin was to induce the release of nascent chains from membrane-bound ribosomes of secreted proteins that were in the process of translocation through a protein conducting channel. Once released, ions could flow through the channel and conductance could be observed. Since cytosolic ribosomes have also been demonstrated to be bound to isolated yeast mitochondria [29], it is possible that mitochondrial-bound ribosomes are somehow involved in puromycin-induced mitochondrial import inhibition. While we can not exclude this possibility completely, two of our results suggest that the involvement of cytosolic ribosomes in the mechanism of puromycin import inhibition is unlikely in the mitochondrial system. First, while ATP can overcome puromycin import inhibition (Fig. 2), it does not alter the drugs effect on protein synthesis. Second, in order for ATP to overcome puromycin import inhibition it must be transported into the mitochondrial matrix (Fig. 2). If puromycin-induced import inhibition was due to a direct interaction with bound cytosolic ribosomes, the effect of ATP in overcoming the inhibition would be expected to act at the outer mitochondrial surface.

Taken together, our results strongly suggest that puromycin inhibits protein import by interfering with an ATP-dependent reaction that takes place inside the inner mitochondrial membrane. It been demonstrated that the intramitochondrial heat shock-like protein, Mhsp70, is involved in the import reaction and that ATP may be involved in its function [30]. Yet, it remains unclear exactly how many different intramitochondrial ATP-dependent reactions are involved in the import process, perhaps in addition to those mediated by molecular chaperones. It is precisely for this reason that our results are useful; because puromycin rather specifically inhibits an ATP-dependent reaction involved in protein import into mitochondria. This finding clearly identifies a very unique ATP-dependent reaction, differentiating it from any number of other unrelated reactions that utilize ATP as well. Consequently, our finding that puromycin inhibits protein import by interfering with an intramitochondrial ATP-dependent reaction is likely to provide a powerful new tool for analyzing the ATP-dependent component(s) involved in the import process in greater detail.

Acknowledgments

We would like to thank Mary Beth Verner and Marie Yablonski for excellent technical help and Drs. R. Scaduto and G. Schatz for critically reading the manuscript. This study was supported by grant DCB-9105609 from the National Science Foundation.

References

1 Attardi, G. and Schatz, G. (1988) Annu. Rev. Cell Biol. 4, 289–333.
2 Douglas, M.G., McCammon, M.T. and Vassarotti, A. (1986) Microbiol. Rev. 50, 166–178.

3 Pfanner, N. and Neupert, W. (1990) Annu. Rev. Biochem. 59, 331–353.
4 Rapoport, T.A. (1986) CRC Crit. Rev. Biochem. 20, 73–137.
5 Chen, W.-J. and Douglas, M.G. (1987) Cell 49, 651–658.
6 Eilers, M., Opplinger, W. and Schatz, G. (1987) EMBO J. 6, 1073–1077.
7 Pfanner, N. and Neupert, W. (1986) FEBS Lett. 209, 152–156.
8 Hwang, S.T. and Schatz, G. (1989) Proc. Natl. Acad. Sci. USA 86, 8432–8436.
9 Nelson, N. and Schatz, G. (1979) Proc. Natl. Acad. Sci. USA 76, 4365–4369.
10 Fujiki, M. and Verner, K. (1991) J. Biol. Chem. 266, 6841–6847.
11 Hurt, E.C., Pesold-Hurt, B., Suda, K., Opplinger, W. and Schatz, G. (1985) EMBO J. 4, 2061–2068.
12 Stueber, D., Ibrahimi, I., Cutler, D., Dobberstein, B. and Bujard, H. (1984) EMBO J. 3, 3143–3148.
13 Vestweber, D. and Schatz, G. (1988) EMBO J. 7, 1147–1151.
14 Eilers, M. and Schatz, G. (1986) Nature 322, 228–232.
15 Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247–256.
16 Daum, G., Bohni, P.C. and Schatz, G. (1982) J. Biol. Chem. 257, 13028–13033.
17 Gasser, S.M., Daum, G. and Schatz, G. (1982) J. Biol. Chem. 257, 13034–13041.
18 Akerman, K.E.O. and Wikstrom, M.K.F. (1976) FEBS Lett. 68, 191–197.
19 Klingenberg, M. (1989) Arch. Biochem. Biophys. 270, 1–22.
20 Buchanan, J.L., Primack, M.P. and Tapley, D.F. (1971) Endocrinology 89, 534–537.
21 Chen, W.-J. and Douglas, M.G. (1988) J. Biol. Chem. 263, 4997–5000.
22 Chirico, W.J., Waters, M.G. and Blobel, G. (1988) Nature 332, 805–810.
23 Pfanner, N., Tropschug, M. and Neupert, W. (1987) Cell 49, 815–823.
24 Sheffield, W.P., Shore, G.C. and Randall, S.K. (1990) J. Biol. Chem. 265, 11069–11076.
25 Verner, K. and Schatz, G. (1987) EMBO J. 6, 2449–2456.
26 Zimmermann, R., Sagstetetter, M., Lewis, M.J. and Pelham, H.R.B. (1988) EMBO J. 7, 2875–2880.
27 Pfanner, N., Pfaller, R., Kleene, R., Ito, M., Tropschug, M. and Neupert, W. (1988) J. Biol. Chem. 263, 4049–4051.
28 Simon, S.M. and Blobel, G. (1991) Cell 65, 371–380.
29 Kellems, R.E., Allison, V.F. and Butow, R.A. (1975) J. Cell Biol. 65, 1–14.
30 Manning-Krieg, U.C., Scherer, P.E. and Schatz, G. (1991) EMBO J. 10, 3273- 3280.