Import of a DHFR Hybrid Protein into Glycosomes In Vivo Is Not Inhibited by the Folate-Analogue Aminopterin

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Abstract. Dihydrofolate reductase fusion proteins have been widely used to study conformational properties of polypeptides translocated across membranes. We have studied the import of dihydrofolate reductase fusion proteins into glycosomes and mitochondria of *Trypanosoma brucei*. As signal sequences we used the last 22 carboxy-terminal amino acids of glycosomal phosphoglycerate kinase for glycosomes, and the cleavable presequences of yeast cytochrome b_2 or cytochrome oxidase subunit IV for mitochondria. Upon addition of aminopterin, a folate analogue that stabilizes the dihydrofolate reductase moiety, import of the fusion protein targeted to glycosomes was not inhibited, although the results of protease protection assays showed that the fusion protein could bind the drug. Under the same conditions, import of a DHFR fusion protein targeted to mitochondria was inhibited by aminopterin. When DHFR fusion proteins targeted simultaneously to both glycosomes and mitochondria were expressed, import into mitochondria was inhibited by aminopterin, whereas uptake of the same proteins into glycosomes was either unaffected or slightly increased. These findings suggest that the glycosomes possess either a strong unfolding activity or an unusually large or flexible translocation channel.

TRYPANOSOMES compartmentalize most of their glycolytic enzymes in a microbody, the glycosome (Opperdoes, 1987). Although the various types of microbodies—glycosomes, peroxisomes, glyoxysomes—differ substantially in enzyme content, they show many similarities and are clearly evolutionarily related (Borst, 1989; Hannaert and Michels, 1994). All their resident proteins are encoded in the nucleus, synthesized on free ribosomes in the cytoplasm and imported posttranslationally across the single boundary membrane of the organelle (Lazarow and Fujiki, 1985; Subramani, 1993). Signals for targeting proteins to peroxisomes have been extensively characterized (reviewed in de Hoop and Ab, 1992). Presently, known peroxisomal targeting signals (PTS)[1] can be separated into two groups. PTS1 consists of the tripeptide SKL or related sequences located at the carboxy terminus of imported proteins (Gould et al., 1989), and PTS2 of an amino-terminal presequence which in some cases is cleaved after import (Osumi et al., 1991; Swinkels et al., 1991). PTS1 is also found in trypanosomes (Blattner et al., 1992; Sommer et al., 1992) and there is evidence for the existence of PTS2 as well (Blattner et al., 1995). In contrast with peroxisomes, where many components of the import machinery have been identified by genetic approaches (Elgersma et al., 1993; Kunau et al., 1993; Spong and Subramani, 1993; Zhang et al., 1993) or examination of naturally occurring mutants such as human Zellweger syndrome (Gartner et al., 1992; Shimozawa et al., 1992), no components of the glycosomal protein import machinery have been identified. Likewise, not much is known about the characteristics of the glycosomal import process. In peroxisomes, import can be separated into a binding and a translocation step and requires ATP (Wendland and Subramani, 1993) but not a membrane potential (Imanaka et al., 1987).

To study the import machinery of glycosomes, we decided to employ methodology already applied to yeast mitochondria. During transit of a polypeptide into mitochondria it must be in an unfolded state. Methotrexate, a folate analogue, binds with high affinity to dihydrofolate reductase (DHFR), stabilizing its folded conformation and thereby inhibiting unfolding. DHFR coupled to a mitochondrial targeting signal can be imported into mitochondria, but upon addition of methotrexate or aminopterin, a membrane-permeable analogue, the stably folded com-

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1. *Abbreviations used in this paper*: b_2, yeast cytochrome b_2; coxIV, yeast cytochrome oxidase subunit IV; DHFR, dihydrofolate reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PTS, peroxisomal targeting signal.

plex gets stuck in the import channel (Eilers and Schatz, 1986; Rassow et al., 1989; Wienhues et al., 1991). Such stuck import intermediates could be photo-cross-linked to components of the import machinery (Vestweber et al., 1989).

In this paper we describe the effect of aminopterin on the import of a DHFR fusion protein targeted to glycosomes. We found no aminopterin-induced import inhibition although extensive controls showed that the drug bound to the hybrid protein in vitro and in vivo. Furthermore we report for the first time the application of the inducible gene expression system for trypanosomes (Wirtz and Clayton, 1995).

Materials and Methods

Plasmid Construction and Generation of Permanent Cell Lines

The DHFR-phosphoglycerate kinase C (PGK-C) fusion protein (DHFR-PGK-C) was constructed the following way: the DHFR coding region with a SacII site at the 3'-end was obtained by PCR (Saiki et al., 1988) using plasmid p9/2 (van Loon et al., 1986) as a template and primer oligonucleotides CZ262 (GTACAAGCTTATGGTTCGACCATTGAACTG) and CZ264 (GATCCCGCGGAGTCTTTCTTCTCGTAGACTT) (The corresponding restriction sites are underlined). The product was digested with HindIII and SacII and cloned upstream of the nucleotides encoding the last 22 amino acids of PGK-C by insertion into HindIII-SacII-digested pJP 62 (Fung and Clayton, 1991), replacing a chloramphenicol acetyltransferase-cassette, yielding pJP 101. DHFR-ΔPGK-C (pJP101s) was made similarly using a pJP 62-variant in which the last five amino acids RWSSL are missing from the PGK-C extension (Blattner et al., 1992). From both vectors, pJP 101 and pJP 101s, SmaI-StuI fragments containing parp- 5'- and 3'-untranslated regions and the DHFR-(Δ)PGK-C fusion were cut out and ligated into pHD 102 that was previously cut with SmaI. The resulting vectors were named p102/101 and p102/101s, respectively. pHD 102 is a variant of the previously described pHD 30 (Janz and Clayton, 1994), in which the luciferase cassette is replaced by a β-tubulin-neomycin-phosphotransferase-cassette (β-tub-npt) (ten Asbroek et al., 1990) and the single PstI-site is converted into a StuI site (C. Hartmann and C. Clayton, unpublished observation). It has a PARP promoter that is separated by a SmaI site from the β-tub-npt-cassette. The fusion between the presequence of yeast cytochrome oxidase subunit IV (pcoxIV) and DHFR was cut out as an EcoRI-HindIII fragment from pDS5/2-1-PCoxIV-DHFR (Hurt et al., 1984), blunted and ligated into blunted HindIII-BamHI-cut (5,493 bp) p102/101, yielding p102/200. p102/250 containing the pcoxIV-DHFR-PGK-C fusion was obtained by ligating an EcoRI-BstXI fragment excised from p102/200 to an EcoRI-BstXI (5,462 bp) fragment from the vector p102/101. By replacing the pcoxIV-cassette of p102/250 by a fragment coding for the first 167 amino acids of yeast cytochrome b_2 made by PCR from Plasmid SR1 (Glick et al., 1993) p102/210 was obtained. p102/210 was cut with BstBI, SpeI, and blunted with mung bean nuclease to remove the sequence coding for the amino acids of position 43 to 67 and generate p102/220 containing Δb_2-DHFR. All open reading frames obtained by PCR and subcloning (also the ones described later in this section) were checked by sequencing.

To generate permanent cell lines, 10 to 100 μg of EcoRI- and StuI-digested plasmid was used to transfect 3×10^7 AnTat 1.1 trypanosomes by electroporation (Beverley and Clayton, 1993). Selection at 5 μg/ml G418 was applied 24 h after transfection and increased in intervals to 10 μg/ml G418 in the following days. Clonal cell lines were obtained by limited dilution of drug-resistant populations.

To obtain cell lines expressing the different DHFR fusions in a tetracycline-inducible fashion, DHFR-cassettes bearing HindIII and BamHI or BglII sites were obtained by excising the fragments from p102/101 or by PCR, using p101/200 and p102/250 as templates and primer oligonucleotide CZ368 (GATCAAGCTTATGCTTTCACTACGTCAATC) with either CZ374 (GAAGATCTTTAGTCTTTCTTCTCGTAGACT) or CZ370 (GATCGGATCCTTAAAGAGAGCTCCACCGGT). These cassettes were subcloned into pHD 430 and transfected into AnTat 1.1 trypanosomes expressing the tetracycline repressor as described in Wirtz and Clayton (1995).

SDS-PAGE, Western Blotting, Antibodies, and Quantification of Immunoblots

Samples were electrophoresed on SDS-PAGE (Laemmli, 1970) and electroblotted onto nitrocellulose according to published methods (Harlow and Lane, 1988). The nitrocellulose filters were blocked for 1 h or overnight in TBS-T (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk powder. Then they were incubated in TBS-T in the presence of the primary antibody for 1 h at room temperature. After washing in TBS-T the filters were incubated with alkaline phosphatase-coupled anti-rabbit IgG as secondary antibody. Detection was performed using chemiluminescence developing reagents (ECL system; Amersham Corp., Arlington Heights, IL). Films were scanned with a scanner equipped with a transparency module (Model Arcus II; AGFA Corp., Orangeburg, NY) and bands were quantified using the NIH-Image software (release 1.56b9). Amounts of antigen and exposure time of films were adjusted to be in a linear range to ensure accurate quantification of protein bands (Zhang et al., 1993). Antibodies used were anti-DHFR (dilution 1:2,000), anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000), anti-hsp60 antibodies (1:2,000) or anti–PGK-C antibodies (1:4,000). The anti-murine DHFR antiserum does not cross-react with the trypanosomal homologue, presumably because the homology of the two proteins is below 20%. The anti–PGK-C antiserum cross-reacts strongly with the highly homologous, cytosolic PGK-B (It is therefore called anti-PGK antiserum for brevity). Glycosomal PGK-C is absent from the procyclic form and therefore not detected in these experiments.

Cell Fractionation of Transformed Cells

Approximately 7×10^8 cells were harvested by centrifugation (700 *g* for 10 min), washed once in HEDS+ buffer (25 mM Hepes-KOH [pH 7.8], 1 mM EDTA, 250 mM sucrose, 1 mM DTT, 1 mM *o*-phenantrolin and 2 μg/ml leupeptin) and resuspended in 200 μl HEDS+ (this corresponds to a 1:1 ratio of pelleted cells and buffer). Silicon carbide was added and the suspension was ground with the aid of a mortar and pestle until more than 90% of the cells were broken. The degree of breakage was checked microscopically. After adding 2.5 ml of HEDS+ the suspension was centrifuged once for 5 min (300 *g*) at 4°C to remove the silicon carbide and twice for 10 min (1,800 *g*) at 4°C to remove the unbroken cells. An organellar pellet was obtained by centrifugation for 30 min at 33,000 *g* (4°C). This pellet was resuspended in 200 μl HEDS+ and layered on top of a linear 1–2 M sucrose gradient that was spun for 90 min at 42,000 rpm in a Beckman SW60 Ti rotor (Beckman Instrs., Inc., Fullerton, CA). 10–12 fractions were collected manually and diluted twofold with HEDS+ before TCA precipitation (Glick, 1991). Fractions were analyzed by SDS-PAGE, Western blot and quantification of the respective bands.

For fractionation of cells with digitonin, 2.5×10^6 cells (10 μg protein) per aliquot were harvested by centrifugation, washed once in trypanosome homogenization buffer (THB), containing 25 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.3 M sucrose, 1 mM DTT and 2 μg/ml leupeptin, and resuspended in 112.5 μl THB (to yield a final concentration of 2×10^7 cells/ml after addition of the detergent). 12.5 μl of digitonin dissolved at different concentrations in distilled water was added. THB with 0.6 M sucrose was used for fractionation if mitochondria were included in the analysis. At 0.3 M sucrose, mitochondria were stable during and after digitonin fractionation, but collapsed upon protease treatment. The mixture was incubated for 2 min at 37°C, vortexed for 10 s and centrifuged (~12,000 *g*) at 4°C for 10 min. The supernatant was TCA precipitated and proteins were analyzed as indicated above. In titration experiments the optimal digitonin concentration was determined for fractionation of glycosomes and mitochondria (0.2 mg digitonin/mg total cellular protein).

Thermolysin Assay

10^6 cells expressing DHFR-PGK-C were harvested, washed, resuspended in 330 μl thermolysin incubation buffer (0.1 M Hepes-HCl [pH 7.5], 2 mM $CaCl_2$, 0.2% Triton X-100 and 2 μg/ml leupeptin) and separated into 30-μl samples. To five of them 1 μl of a 250 μM stock of aminopterin to a final concentration of 8.3 μM was added. All samples but one received 3 μl of serially diluted thermolysin solution to obtain the desired amount of pro-

tease. Samples were incubated for 30 min at 43°C. The protease was stopped by adding 5 μl of 0.2 M EGTA. Proteins were TCA precipitated, and subsequently analyzed by SDS-PAGE and Western blot.

Killing Curves of Trypanosomes Incubated in Medium Containing Aminopterin

Several aliquots of wild-type cells were diluted to the same density and received aminopterin (dissolved in 0.1 mM $NH_4^+OH^-$) in varying concentrations or the same amount of 0.1 mM $NH_4^+OH^-$ without the drug. Cell densities were determined every 24 h by counting. The minimal concentration which impaired growth was 5 μM. To test whether the toxic effect of aminopterin on trypanosomes could be reversed, 100 μM thymidine was added to the medium (Beck and Ullman, 1990).

In Vivo Import Assays

Cells containing the genes encoding the different fusion proteins under tetracycline control were preincubated for 2–14 h in the presence or absence of 500 μM aminopterin and 30 mM sulfanilamide. The expression of the fusion protein was then induced by adding 2 μg/ml tetracycline. After 120–300 min incubation at 27°C the viability of cells was checked by microscopic examination, cycloheximide was added to 300 μg/ml and 1×10^7 cells per aliquot were harvested or incubated for another hour (chase) before harvesting, washed and resuspended in 450 μl THB (with 0.6 M sucrose for mitochondrial analysis and supplemented with 100 μM aminopterin for cells grown in the presence of the drug) and subjected to digitonin fractionation (final concentration of 2×10^7 cells/ml). Pellets were resuspended in 500 μl THB (0.6 M sucrose and +/− 20 μM aminopterin, but without leupeptin). One aliquot (pellet and supernatant) of each assay received proteinase K or trypsin at 20 μg/ml (in experiments with only glycosomes) or 10 μg/ml (in experiments with glycosomes and mitochondria) and was incubated for 30 min at 4°C. Control aliquots received 0.2% Triton X-100 and 0.2% deoxycholate. The protease was stopped by adding PMSF to 1 mM and incubating for 10 min on ice. Trypsin-containing samples received in addition 100 μg/ml soybean trypsin inhibitor. Then the organelles were isolated by centrifugation (4°C) at 12,000 *g* for 10 min. The supernatants and detergent-containing samples were TCA precipitated and proteins were analyzed by SDS-PAGE, Western blot and quantification of the respective bands.

Pulse Experiments

Cells constitutively expressing the DHFR-PGK-C fusion protein were grown in the presence or absence of aminopterin and sulfanilamide for up to 16 h, harvested, washed and resuspended at 10^7 cells/ml in MEM-medium without methionine. [^{35}S]methionine (1,000 Ci/mmol) was added to a final concentration of 0.25 μCi/ml and cells were incubated for 30 min at 27°C. At the end of the incubation the viability of the cells was checked by microscopic examination, then a 50,000-fold excess of cold methionine (to 0.5 mg/ml) and cycloheximide to 300 μg/ml were added. Cells were harvested and treated as described above. After stopping the protease digestion all samples were TCA precipitated, washed in cold 95% acetone and resuspended in 100 μl 25 mM Tris-HCl (pH 7.4)/1% SDS as described by Fujiki and Verner (1993). The processed samples were then diluted to 1 ml with TNET (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) supplemented with 2% nonfat dried milk. Samples were precleared by adding 100 μl of Protein A-coupled beads (Protein A Sepharose 6MB; Pharmacia) and incubating for 30 min at 4°C on a rotary shaker. The beads were removed by centrifugation and the supernatant was incubated with 5 μl of specific anti-serum for 1 h and then for another hour with 100 μl Protein A-coupled beads. After a short centrifugation, the pellet was washed four times in TNET, resuspended in 30 μl SDS-PAGE loading buffer, heated for 5 min to 95°C and centrifuged. The supernatant was then analyzed by SDS-PAGE and exposing the salicylate-soaked (in 1 M salicylate, 15 min), dried gels to phosphorimager plates. Bands were quantified using a Fuji BAS 1000 phosphorimager.

Immunoelectron Microscopy

Trypanosomes were fixed with 2% formaldehyde and 0.05% glutaraldehyde. After 1 h on ice, cells were washed, embedded in agarose, dehydrated with ethanol at progressively lowered temperatures and embedded in Lowicryl HM20 at −35°C (Carlemalm et al., 1982). Ultrathin sections were treated with 1% milk powder, 0.5% bovine serum albumin in phosphate-buffered saline (pH 7.4) to block non-specific binding sites and labeled by incubation with primary antibodies (anti-DHFR rabbit serum 1:20, anti-aldolase rabbit serum 1:20 or anti-hsp60 rabbit serum 1:20) and protein A–13-nm gold or 6-nm gold complexes. Double labeling experiments were performed by first labeling one side of sections floating freely on drops of anti-aldolase or anti-hsp60 serum and protein A–6-nm gold complexes. After mounting these sections on Pioloform and carbon-coated grids with the labeled site directed to the grid surface, the upper side of the sections was incubated with anti-DHFR serum and protein A–13-nm gold complexes. After staining with uranyl acetate and lead citrate, sections were examined in a Philips 201 electron microscope at 60 kV.

Controls (not shown): omitting the first antibodies or labeling of wild-type trypanosomes with anti-DHFR serum did not result in any glycosomal or mitochondrial labeling. The specificity of the two side labeling was confirmed by shadowing mounted sections with platinum/carbon. All the large, but not the small gold particles showed a typical shadow, demonstrating that the differently sized gold particles were located on opposite faces of the section (Steverding et al., 1994).

Results

Construction of Stable Cell Lines Expressing Different Versions of Dihydrofolate Reductase Fusion Proteins and Determination of Their Subcellular Localization

We chose to study the import of a hybrid protein consisting of the last 22 amino acids of phosphoglycerate kinase (PGK-C), which has been shown to target chloramphenicol acetyltransferase to glycosomes (Fung and Clayton, 1991), fused to the carboxy terminus of mouse DHFR (DHFR-PGK-C). Stable cell lines were constructed that expressed DHFR-PGK-C or an import-deficient version (DHFR-ΔPGK-C) lacking the last five amino acids (RWSSL, the signal that was shown to be both sufficient and necessary for glycosomal targeting [Blattner et al., 1992]). The subcellular location of the fusion protein was determined by subjecting cells expressing DHFR-PGK-C or DHFR-ΔPGK-C to fractionation with digitonin. Digitonin differentially solubilizes membranes according to their cholesterol content. At low concentrations only the plasma membrane is permeabilized, whereas internal membranes stay intact. With increasing concentrations of detergent, the internal organelles start to release their content (Schulz, 1990; Sommer et al., 1992; Zhang et al., 1993). Thus the truncated DHFR fusion protein, expected to be in the cytoplasm, was released into the supernatant at the same digitonin levels as PGK-B, an endogenous cytosolic marker (Fig. 1 *a*). DHFR-PGK-C, targeted to the glycosome, was released only at higher detergent concentrations. GAPDH, a glycosomal marker, was released at yet higher digitonin concentrations than DHFR-PGK-C. The difference in the solubility of DHFR-PGK-C and GAPDH is probably due to the fact that the artificial fusion protein is not assembled into the glycosomal core, a complex consisting of the endogenous glycosomal proteins (Misset et al., 1986). Similarly, different peroxisomal matrix proteins were released at varying digitonin concentrations in yeast mutants defective in peroxisome biogenesis (Zhang et al., 1993). Fig. 1 *b* shows a Western blot of pellet and supernatant fractions of trypanosomes expressing either DHFR-PGK-C or the truncated version. The digitonin concentration used (0.2 mg/mg) was employed in all later experiments. At this concentration, glycosomal DHFR-PGK-C is located in the pellet and protease protected, whereas DHFR-ΔPGK-C is located in the supernatant and digested by pro-

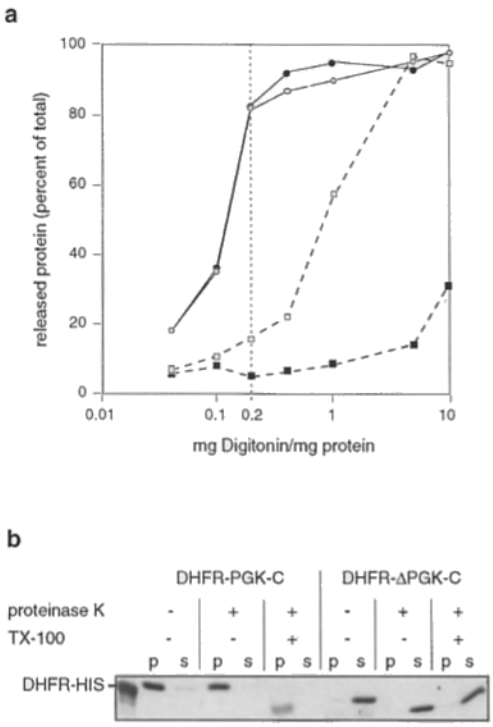

Figure 1. (*a*) Digitonin fractionation of cells expressing DHFR-ΔPGK-C or DHFR-PGK-C, respectively. Cells expressing either of the two constructs were treated with increasing amounts of digitonin and separated into an organellar pellet and a cytoplasmic supernatant. Fractions were analyzed by measuring the intensities of the respective bands on Western blots. The percentage of protein contained in the supernatant relative to the total amount was plotted against the concentration of digitonin. The data shown for GAPDH and PGK-B are derived from the fractionation of the cell line expressing DHFR-ΔPGK-C; corresponding data from the DHFR-PGK-C cell line were similar. —○—, DHFR-ΔPGK-C; --□--, DHFR-PGK-C; --■-- , GAPDH; —●—, PGK-B. The dashed line indicates the digitonin concentration that was used for all subsequent fractionation experiments. (*b*) Western blot of cells expressing either DHFR-PGK-C or DHFR-ΔPGK-C fractionated with 0.2 mg Digitonin per mg cellular protein. The glycosomal DHFR-PGK-C is located in the pellet fractions (*p*) and the cytosolic DHFR-ΔPGK-C in the supernatant fractions (*s*). DHFR-PGK-C is inside the organelle and therefore protected against protease, unless Triton X-100 is added. DHFR-ΔPGK-C is accessible to protease and the PGK-C extension is cleaved off to yield the partially protease-resistant DHFR moiety (see also Fig. 3).

tease to its partially protease-resistant DHFR moiety (see below).

The glycosomal location of the DHFR-PGK-C was confirmed by immunoelectron microscopy. On sections of cells expressing the fusion protein almost all DHFR-PGK-C as well as the glycosomal marker aldolase were found inside the glycosomes (Fig. 2 *b*). Some weak background staining of the nucleus was due to cross-reactivity of the anti-DHFR serum, since it was detectable with wild type cells as well (not shown). To exclude any localization of DHFR-PGK-C in mitochondria, sections of the same cells were stained for DHFR-PGK-C and hsp60, a mitochondrial marker (Fig. 2 *a*). None of the organelles that showed staining for hsp60 showed any reactivity with the anti-DHFR serum, confirming the unique glycosomal location of DHFR-PGK-C. On sections of cells expressing the truncated fusion protein all of it was found in the cytoplasm (not shown).

Binding of Aminopterin to DHFR-PGK-C in Whole Cell Lysates

A necessary prerequisite for subsequent experiments was to show that the DHFR-PGK-C hybrid protein expressed by the trypanosomes was still able to bind aminopterin. For that purpose we performed a protease protection assay as described by Eilers and Schatz (1986). Lysates of cells expressing DHFR-PGK-C were preincubated with or without aminopterin (8.3 μM) before thermolysin, a bacterial protease, was added in increasing concentrations. After terminating the reactions they were analyzed by Western blot (Fig. 3). In lane 1 the undigested form is shown (addition of aminopterin did not change the migration behavior of the construct [not shown]). In the absence of aminopterin (lanes *7–11*) the fusion protein is completely degraded. In the presence of the drug (lanes *2–6*) a partially degraded product is observed. The size of the products suggests that the protease cleaves off the unprotected PGK-C extension, leaving the DHFR moiety intact because it is protected by the bound aminopterin. A similar phenomenon was observed by Eilers and Schatz (1986). Cleavage of the extension shows that thermolysin is active in the presence of aminopterin, an observation further confirmed by complete digestion of albumin by thermolysin in the presence of 8.3 μM aminopterin.

Permeability of Trypanosomes to Aminopterin

To bind to DHFR-PGK-C in vivo, the aminopterin must be able to penetrate the trypanosomes. To test this, we made use of the fact that aminopterin, as a folate analogue, is toxic for trypanosomatids, since it binds to the endogenous DHFR (Kaur et al., 1988) and thereby inhibits pyrimidine synthesis (and other metabolic pathways). Wild-type cells cultivated in medium containing 5 μM aminopterin ceased multiplying immediately and died after one to two days, confirming that the drug was entering the cells (Fig. 4). 5 μM of aminopterin was also lethal for cells expressing the DHFR-PGK-C fusion protein (not shown). By adding thymidine to the growth medium, the toxic effect of aminopterin can be partially reversed (Beck and Ullman, 1990). Trypanosomes cultured in both aminopterin and thymidine exhibited a near-normal growth rate in the first few days. In all subsequent experiments we used 500 μM aminopterin, a 100-fold excess.

Analysis of the Effect of Aminopterin on Import of DHFR-PGK-C into Glycosomes In Vivo

To determine the effect of aminopterin on the import of DHFR-PGK-C into glycosomes in vivo, we used trypanosomes expressing the protein under control of a tetracycline-inducible promoter. Cell lines containing the fusion protein constructs were preincubated for 2 h with or without 500 μM aminopterin. (In most experiments 30 mM sulfanilamide was also added to reduce the intracellular levels of dihydrofolate that might compete with aminopterin

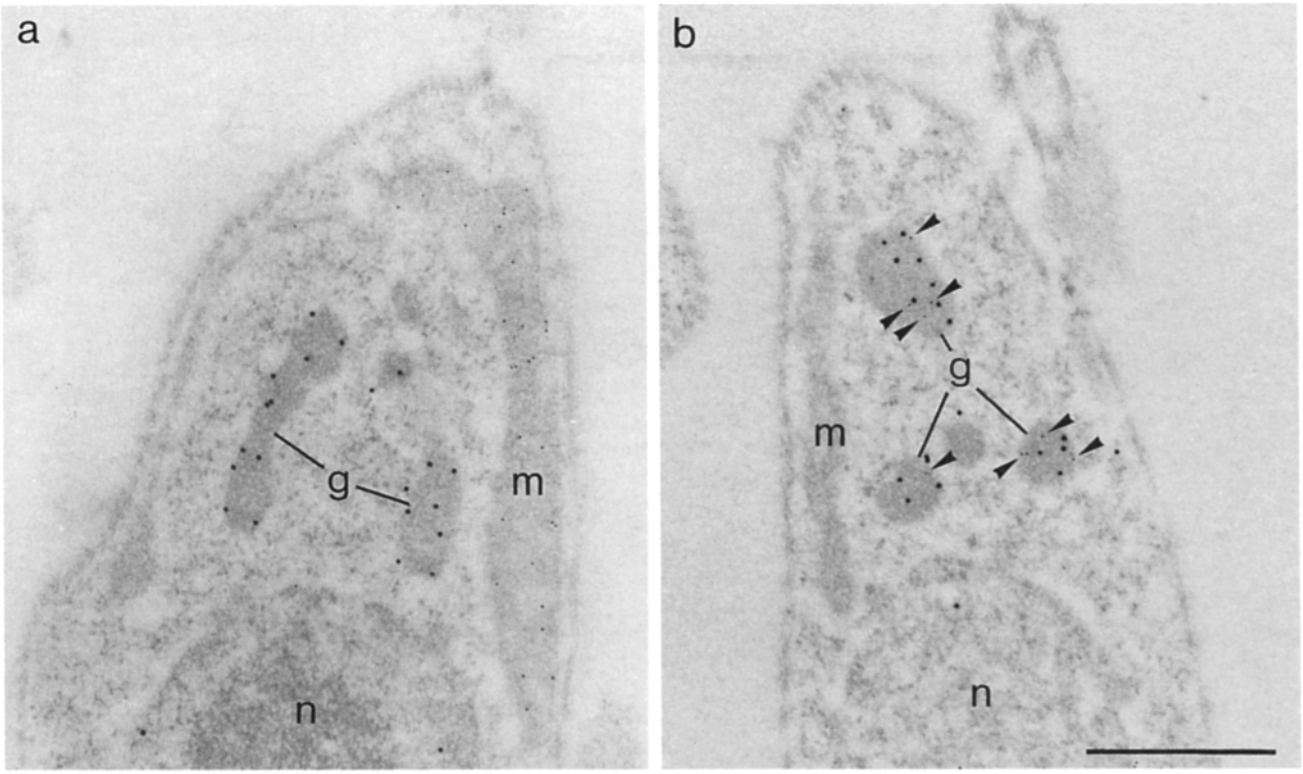

Figure 2. Cellular localization of DHFR-PGK-C by on-section labeling of T. brucei procyclic forms embedded in Lowicryl HM20. Sections were probed on one side with anti-hsp60 (*a*) or anti-aldolase (*b*) rabbit serum and protein A–6-nm gold and on the other side with anti-DHFR rabbit serum and protein A–13-nm gold. The 13-nm gold particles colocalize only with the aldolase label (*b*, 6 nm gold, *arrow heads*) in the more electron dense and spherical glycosomes (*g*), but not with the hsp60 label (*a*, 6 nm gold) in the brighter and elongated mitochondrion (*m*). *n*, nucleus. Bar, 0.5 μm.

[Wienhues et al., 1991].) The expression of DHFR-PGK-C was induced by adding 2 μg/ml tetracycline. After 2–5 h protein expression was stopped by adding 300 μg/ml cycloheximide and the cells were harvested. An organellar pellet and a cytoplasmic supernatant were obtained by fractionation with 0.2 mg digitonin/mg total cellular protein, and the location of the newly synthesized protein assessed by Western blot (Fig. 5). No processing of any glycosomal protein upon import has been detected so that inaccessibility to externally added protease is the only available biochemical criterion for completed import (Sommer et al., 1990). The DHFR-PGK-C hybrid protein was expressed (lane *1*) and fully resistant to proteinase K (lane *2*) or trypsin (not shown), indicating its complete import into glycosomes; no DHFR-PGK-C was detected in the cytoplasmic fractions (lanes *1'* and *2'*). Addition of detergent to the organellar pellet rendered the fusion protein protease-sensitive and its PGK-C extension was cleaved off (lane *3*, compare with Fig. 3), confirming that the chosen conditions were suitable to assess for import. Under these conditions, GAPDH is not digested, because most glycosomal proteins are very protease-resistant (Clayton,

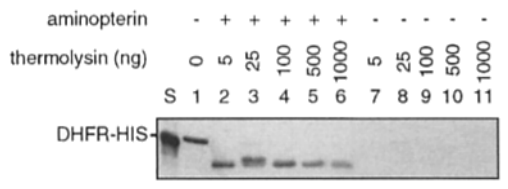

Figure 3. Aminopterin protects DHFR-PGK-C from digestion by thermolysin. Lysates of cells expressing DHFR-PGK-C were incubated in the presence or absence of aminopterin with increasing amounts of thermolysin. After stopping the protease reaction samples were analyzed by SDS-PAGE and Western blot. As a size standard, bacterially expressed and affinity-purified histidine-tagged DHFR (DHFR-HIS) was run in lane *S*.

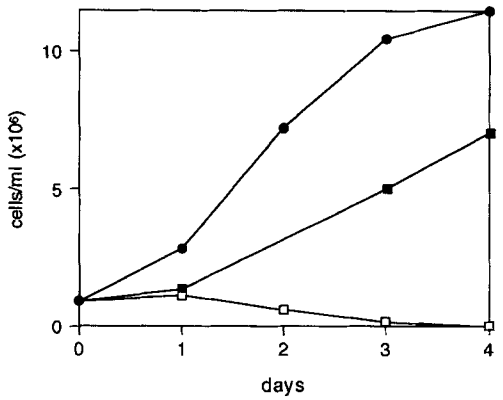

Figure 4. Effect of aminopterin on the growth of wild-type trypanosomes. Cells were cultivated in the absence (—●—) or presence (—□—) of 5 μM aminopterin. This amount of drug effectively kills the trypanosomes within 1–2 d. If 100 μM thymidine is supplemented to the medium (—■—), cell growth is restored to near-normal rates for the first few days.

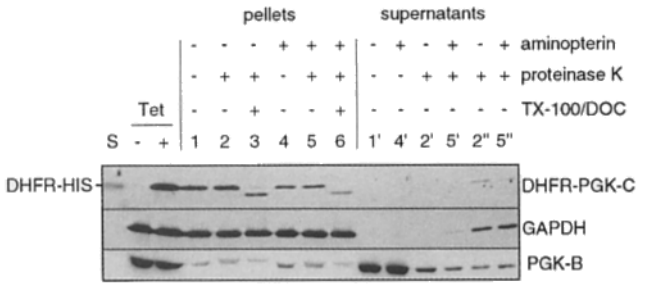

Figure 5. Effect of aminopterin on the import of DHFR-PGK-C into glycosomes. Cells were incubated for 2 h in the presence or absence of aminopterin and sulfanilamide, before the fusion protein was induced for 3 h with tetracycline. Then the cells were separated into pellet- (lanes *1–6*) and supernatant fractions (lanes *1′–5′*). Two aliquots each were treated with protease, one of which received 0.2% Triton X-100 and 0.2% deoxycholate (*DOC*) as a control. Lanes *2″* and *5″* are the supernatants of the protease-treated fractions after reisolation of the organelles. All fractions were immunoblotted with antibodies against mouse DHFR, GAPDH as a control for the integrity of the organelles and, as a control for efficient fractionation, PGK (PGK-B). *S*, bacterially expressed DHFR-HIS as standard; *Tet*, tetracycline; *−/+*, lysates of uninduced and constantly induced cells, respectively.

1987; Sommer et al., 1990). The cytosolic marker PGK-B was only partially digested under these conditions. To our surprise, the presence of aminopterin throughout the period of fusion protein synthesis had no major effect on import. All the DHFR-PGK-C was located in the organellar fraction and protease-protected (lanes *4–6*). Only upon reisolation of glycosomes after the protease treatment could a very faint signal be detected in the supernatant (lane *5″*), but this appeared to be due to contamination with glycosomes as is indicated by the presence of GAPDH in the same fraction. The same slight contamination was detected in samples without aminopterin treatment (lane *2″*). In contrast to yeast (Wienhues et al., 1991), aminopterin and sulfanilamide lowered the expression of proteins in trypanosomes by ~50% (compare strength of bands in lanes *1* and *4*), but control experiments revealed no inhibition of glycosomal import of endogenous proteins (not shown). Other experiments that were performed with a variety of different incubation times and aminopterin concentrations gave similar results.

Since the induction kinetics of genes expressed under the control of tetracycline operators is slow by comparison with the kinetics of import of glycosomal proteins (Wirtz and Clayton, 1995; Borst, 1989), pulse experiments with [^{35}S]methionine were performed. The design of the experiments was basically the same as above; cells were preincubated with aminopterin (and in some experiments sulfanilamide) then pulse labeled with [^{35}S]methionine for up to 30 min. After cell fractionation and protease digestion DHFR-PGK-C, and aldolase as an internal control, were immunoprecipitated and analyzed by SDS-PAGE and fluorography. If a genuine import intermediate were to be generated and stuck across the glycosomal membrane, import sites should become jammed, thereby inhibiting subsequent import of more polypeptides. As a consequence, the amount of imported, protease-protected product should decrease and a backup of precursor proteins in the cytoplasm should be observed. This situation was indeed found in the case of mitochondria (Vestweber and Schatz, 1988*a*; Rassow et al., 1989; Wienhues et al., 1991). In Fig. 6 the quantification from four experiments is shown. Although upon incubation of cells with aminopterin a slight decrease of protease-protected (*left*) and of pellet-associated (*right*) material occurred, statistical analysis of the data (Wilcoxon-Mann-Whitney test [$\alpha = 0.05$]) indicated that the differences were not significant. In the same experiments no inhibitory effect of aminopterin on the import of endogenous aldolase (which probably has a PTS2) could be detected. Since there is evidence that peptides carrying a PTS1 can inhibit import of a protein with a different targeting signal into peroxisomes (Walton et al., 1992*b*), thereby suggesting a common downstream component of the import machinery, this is a further indication that no significant number of import sites was blocked by drug-induced import intermediates.

The distribution of anti-DHFR label in trypanosomes in which expression of the fusion protein was induced in the presence or absence of aminopterin was also compared by immunoelectron microscopy. The distributions in the presence and absence of drug were indistinguishable; and there was no evidence for accumulation of fusion protein near the glycosomal membrane (not shown). This further confirmed that the drug had no effect on import.

Aminopterin-induced Inhibition of Protein Import into Mitochondria but not into Glycosomes

So far we had detected no inhibition of import of a DHFR-containing fusion protein into glycosomes by ami-

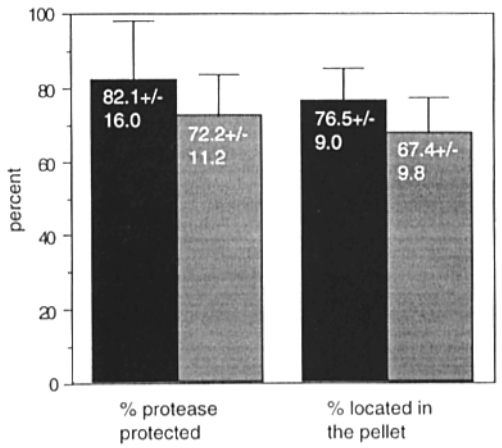

Figure 6. Lack of an aminopterin-induced inhibition of DHFR-PGK-C import into glycosomes. Cells were preincubated for 2–14 h with 500 μM aminopterin and 30 mM sulfanilamide. After a 30-min pulse with [^{35}S]methionine cells were harvested and fractionated with digitonin into a crude glycosomal pellet and a cytoplasmic supernatant. Half of each fraction was treated with proteinase K for 30 min. DHFR-PGK-C and aldolase (not shown) were immunoprecipitated and analyzed by SDS-PAGE, fluorography and quantification on a phosphorimager. The percentage of fusion protein present in the protease-treated glycosomal fraction compared to the untreated glycosomal fraction was calculated (*left*). On the right the percentage of fusion protein present in the glycosomal pellet relative to the cytosolic supernatant is shown. ■ without and ■ with aminopterin, respectively.

nopterin, but although DHFR-PGK-C bound the drug in cell lysates, we could not rule out the possibility that in vivo binding of the drug to the fusion protein did not occur. To prove that the aminopterin could indeed bind in vivo, we expressed two more types of chimeric proteins in trypanosomes. One consisted of a mitochondrial targeting signal fused to the amino-terminus of the DHFR: the accumulation of an aminopterin-induced mitochondrial import intermediate would demonstrate binding of the drug to the DHFR moiety in vivo. The other consisted of a tripartite fusion protein consisting of the same mitochondrial signal sequence fused to the amino terminus of DHFR-PGK-C. The resulting polypeptide should have targeting capability for both glycosomes and mitochondria and therefore be imported into both organelles. Since aminopterin inhibits import of DHFR hybrid proteins into (yeast) mitochondria in vivo (Wienhues et al., 1991) but apparently not into glycosomes, a shift of the distribution of fusion protein between the two organelles towards the glycosome could be expected upon addition of the drug. Such a redistribution would additionally demonstrate binding of aminopterin to the DHFR moiety in trypanosomes in vivo.

As far as we knew, no mitochondrial targeting signal in trypanosomes had been functionally characterized, although the existence of matrix targeting signals could be predicted on the basis of sequence homologies (Effron et al., 1993; Peterson et al., 1993; Clayton et al., 1995). In analogy to the experiments of Wienhues and colleagues (1991) in yeast, we decided to test the extensively characterized presequences of yeast cytochrome b_2 (b_2) (van Loon et al., 1986) and yeast cytochrome oxidase subunit IV (Hurt et al., 1984) for function in trypanosomes. We constructed stable cell lines expressing DHFR hybrid proteins bearing these NH_2-terminal presequences. The cytochrome oxidase subunit IV presequence used consists of the first 22 amino acids and a linker of six amino acids (Hurt et al., 1984, 1985; Horwich et al., 1985). The signal for the Δb_2-DHFR consists of the first 167 amino acids (Glick et al., 1993) of the precursor protein with an internal deletion of 25 amino acids, which abolishes its intermembrane space targeting ability (Beasley et al., 1993; Schwarz et al., 1993) so that the mutated presequence functions only as a matrix-targeting signal. Both fusion proteins were imported and processed. Δb_2-DHFR was processed once from ~38 kD to ~34 kD, which coincides well with the predicted sizes of the mature (37.7 kD) and processed (34.0 kD) forms (Fig. 7 *a*, lane *1*). All forms of the fusion protein were located in an organellar fraction, but only the processed form was protected from externally added protease (lane *2*). Similar results were obtained for pcoxIV-DHFR. The mitochondrial locations of Δb_2-DHFR and pcoxIV-DHFR were confirmed by immunoelectron microscopy with identical results; those for pcoxIV-DHFR are shown in Fig. 8 *a*.

Import and processing of the DHFR bearing the heterologous Δb_2 targeting sequence was characterized in more detail. Import was rather slow: after 3-h induction only ~50% of the fusion protein was imported (Fig. 7 *b*, black bar in lanes *−chase*); increasing to 80% after a 1-h chase (incubation with cycloheximide) (Fig. 7 *a*, lane *1* and Fig. 7 *b*, black bar in lanes *+chase*). Aminopterin reduced the proportion of imported, processed protein and also caused

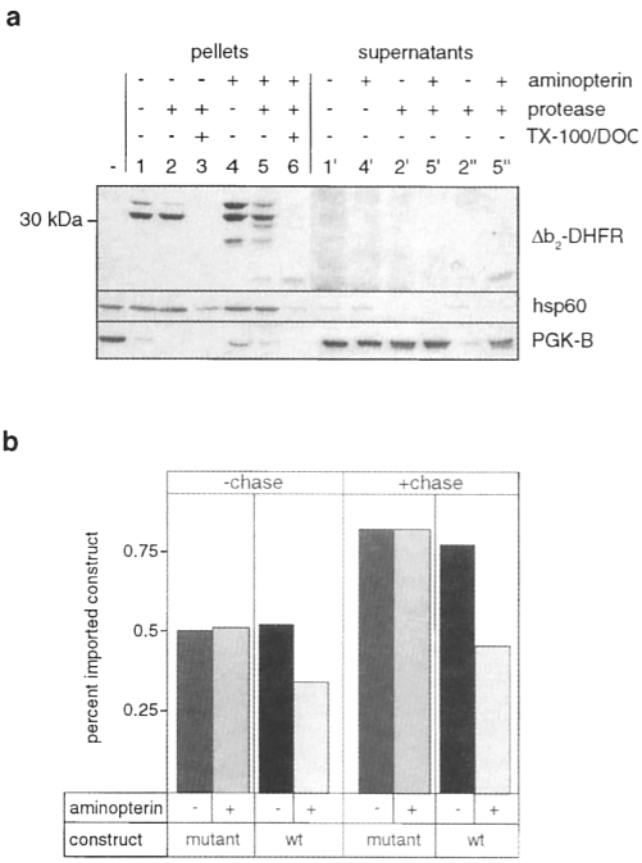

Figure 7. Import of Δb_2-DHFR into mitochondria and generation of an aminopterin-induced import intermediate in trypanosomes. Cells were incubated for 90 min in the absence (*a*, lanes *1–3*; *b*, *black bars*) or presence (*a*, lanes *4–6*; *b*, *white bars*) of aminopterin (sulfanilamide was added to both aliquots of cells), before the fusion protein was induced for 3 h with tetracycline. One half of the cells received 300 μg/ml cycloheximide to inhibit protein synthesis (*a*, *+chase*; *b*, *+chase*) and was incubated for another hour; the other half was immediately processed (*a*, *−chase; b*, *−chase*): cells were separated into pellet (lanes *1–6*) and supernatant fractions (lanes *1′–5′*). Two aliquots each were treated with protease, one of which received 0.2% Triton X-100 and 0.2% deoxycholate (*DOC*) as a control. Lanes *2″* and *5″* are the supernatants of the protease-treated fractions after reisolation of the organelles. All fractions were immunoblotted with antibodies against mouse DHFR, GAPDH, hsp60 and PGK (*PGK-B*). The part of the blot showing the supernatants was exposed longer to show the partially protease-resistant DHFR moiety that is cleaved off from the stuck constructs (lane *5″*). *Tet*, tetracycline; −, lysate of uninduced cells. Import and processing of a mutated, destabilized DHFR fusion protein was not affected by the addition of aminopterin (*b*, *dark shaded bars*, without aminopterin; *light shaded bars*, with aminopterin).

accumulation of both unprocessed and processed forms in a protease-susceptible location. Without chase, ~30% of the construct was imported and processed (Fig. 7 *b*, white bar in lane *−chase*); after 1-h chase this increased only to 45% (Fig. 7 *a*, lane *4* and *b*, white bar in lane *+chase*). This import inhibition is similar to that found in yeast in vivo (Wienhues et al., 1991). The susceptibility of unprocessed protein to protease (compare Fig. 7 *a* with Figs. 4 and 5), indicated that it was stuck across or on the membranes.

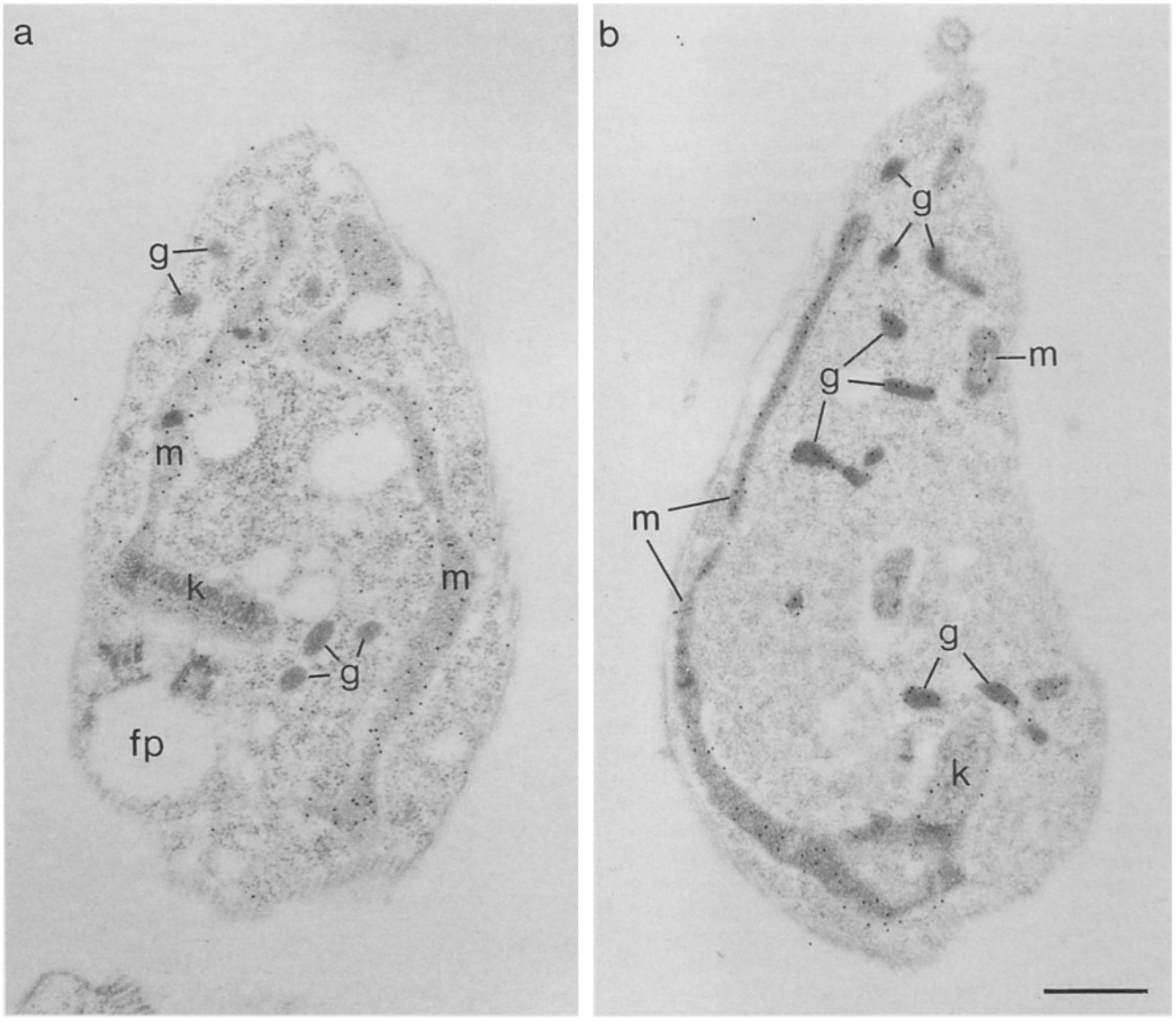

Figure 8. (*a*) Cellular localization of pcox IV-DHFR in the elongated mitochondrion (*m*). Lowicryl HM20 sections were labeled with anti-DHFR serum and protein A–13-nm gold. (*b*) Cellular localization of pcox IV-DHFR-PGK-C in glycosomes (*g*) and mitochondrion (*m*). Lowicryl HM20 sections were labeled with anti-DHFR serum and protein A–13-nm gold. *fp*, flagellar pocket; *k*, kinetoplast. Bar, 0.5 μm.

About 50 amino acids are sufficient to span both mitochondrial membranes (Rassow et al., 1990). Thus the precursor protein should be capable of being processed by the mitochondrial processing protease even while part of the Δb_2-polypeptide and the DHFR moiety is still outside (and protease-accessible). Confirming this prediction, in the presence of aminopterin, some of the processed form was indeed accessible to protease (Fig. 7, lanes *4* and *5*). The cleaved-off DHFR moiety, which is partially protease-resistant, could be detected in the supernatant of the reisolated, protease-treated organellar fraction (lane *5''*, longer exposure). Since mitochondria proved to be more fragile in the presence of protease than glycosomes, we reduced the protease concentration to 10 μg/ml proteinase K or trypsin. Under these conditions, the cytosolic marker PGK-B is largely protease resistant (compare lanes *1'–5'*).

To rule out that the build-up of unprocessed Δb_2-DHFR in the presence of aminopterin was due to the inhibition of the mitochondrial processing protease by aminopterin, we checked import in the absence or presence of aminopterin of a mutated form of the fusion protein, in which a serine replaces cysteine 7 of the DHFR moiety. This mutant is destabilized and is unable to bind aminopterin (Vestweber and Schatz, 1988*b*). Addition of aminopterin should therefore not affect the import of this mutant hybrid protein into mitochondria. As expected, neither import nor processing of the mutant protein were affected by aminopterin (Fig. 7 *b*, *+/−chase, shaded bars*).

We also checked the generation of an aminopterin-induced import intermediate by immunoelectron microscopy. Cells which were induced and grown for 12 h in the presence of aminopterin accumulated Δb_2-DHFR at the mitochondrial membrane: ~62% of the label was present at the mitochondrial membrane and ~38% in the mito-

chondrial lumen, whereas in the absence of the drug ~75% of the label was concentrated in the lumen (not shown). These figures are in good agreement with the ones obtained biochemically after 3 h of induction.

Taken together, these experiments show that, as in yeast, an aminopterin-induced mitochondrial import intermediate of a DHFR fusion protein can be generated in trypanosomes in vivo. This in turn proves that the murine DHFR moiety can bind aminopterin in trypanosomes in vivo.

In the next step we constructed cell lines expressing (under control of a tetracycline-inducible promoter) hybrid proteins consisting of Δb_2-DHFR or pcoxIV-DHFR fused to the last 22 amino acids of PGK-C (Δb_2-DHFR-PGK-C or pcoxIV-DHFR-PGK-C). Both fusion proteins had the desired dual targeting capabilities. Fig. 8 *b* is an electron micrograph of a cell expressing pcoxIV-DHFR-PGK-C: the hybrid protein is present in both organelles. (Unfortunately the levels of Δb_2-DHFR-PGK-C expression in several cell lines were insufficient to allow clear immunoelectronmicroscopical localization.) Analysis of both cell lines by Western blot showed that in the steady-state two forms of the protein were present; the results shown (Figs. 9 and 10) are for Δb_2-DHFR-PGK-C, where the two bands have sizes of ~40 and 36 kD (Fig. 10, lane +). The larger polypeptide is probably identical with the unprocessed hybrid proteins (predicted molecular mass 39.8 kD), and the smaller one (~36 kD) with the form that has been processed by the mitochondrial processing protease. In Fig. 9 a sucrose-gradient fractionation of the same cell line is shown. The smaller, processed form of the hybrid protein is mostly present in the supernatant fraction of the broken-cell suspension as is the mitochondrial marker hsp60 (Fig. 9 *a*, *right*). Apparently, the mitochondrion, which forms an elaborate tubular network in procyclic trypanosomes, is disrupted by the relatively harsh homogenization and thereby releases its contents. The larger form of Δb_2-DHFR-PGK-C cofractionates with the glycosomal marker GAPDH indicating its location in the glycosomes (*left*). The small, spherical glycosomes are more resistant to the silicon carbide treatment than mitochondria and mostly migrate to the expected density of around 1.23 g/cm^3. Only a small portion is disrupted, and therefore some glycosomal content is present in the supernatant. In addition to the two bands observed in Fig. 10 (lane +), a slightly slower migrating protein-species than the processed polypeptide was apparent. It cofractionated clearly with the glycosomal marker, but accurate quantification was impossible, because the doublet-bands were not sufficiently resolved (see also below and Fig. 11). These results are consistent with our conclusion that in the steady-state most of the slower-migrating DHFR species is glycosomal and most of the processed form is mitochondrial.

We now induced expression of the two different hybrid proteins for 3 h in the presence or absence of aminopterin. When the pcoxIV-DHFR-PGK fusion protein was expressed in the absence of aminopterin, 56 ± 2.4% was in the processed form. With aminopterin, this proportion was reduced to 38 ± 2.4%, with a corresponding increase in the amount of full-length protein. (Results are mean ± standard deviation for five experiments.) This result implies inhibition of mitochondrial import by the drug. The distribution of DHFR fusion protein 24 h after induction in the presence or absence of aminopterin was confirmed by immuno-electronmicroscopy (not shown). As before (Fig. 8 *b*), pcoxIV-DHFR-PGK-C was directed to both mitochondria and glycosomes; in the aminopterin-treated samples (not shown) the distribution was similar but there was some evidence for redistribution of some of the mitochondrial label towards the mitochondrial membranes.

The effects of aminopterin on Δb_2-DHFR-PGK-C were more dramatic and were therefore studied in more detail. Results for one experiment are shown in Fig. 10; results for a further five experiments are stated in parentheses as mean and standard deviation. In the absence of drug, ~55% (62 ± 6.5%) of the Δb_2-DHFR-PGK-C was in the

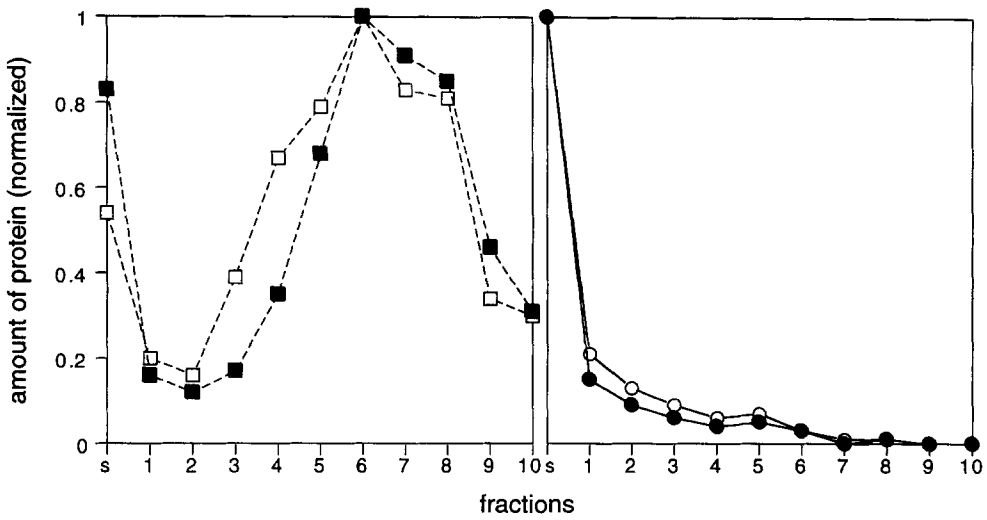

Figure 9. Fractionation of cells expressing Δb_2-DHFR-PGK-C. Cells were broken by grinding with silicon carbide. A granular fraction derived by centrifugation of the broken-cell suspension was separated on a linear 1–2 M sucrose gradient. 10 fractions were collected and analyzed by measuring the intensities of the bands representing the unprocessed and the processed fusion protein on Western blots. The depicted values are normalized using the peak fraction as the standard. *s*, supernatant of broken-cell suspension; 1 (*top*)–10 (*bottom*), collected fractions; —○—, Δb_2-DHFR-PGK-C (processed); --□--, Δb_2-DHFR-PGK-C (unprocessed); —●—, hsp60; --■--, GAPDH.

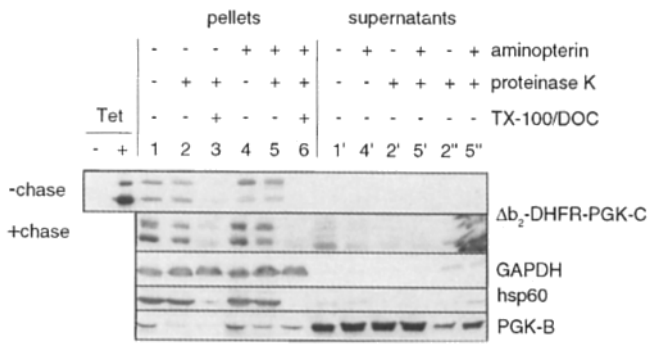

Figure 10. Effect of aminopterin on the import of Δb_2-DHFR-PGK-C into glycosomes and mitochondria. Cells were incubated for 90 min in the presence or absence of aminopterin, before the fusion protein was induced for 3 h with tetracycline. After that, one half of the cells received 300 μg/ml cycloheximide and was incubated for another hour (*+chase*), the other half was processed immediately (*−chase*). Cells were separated into pellet (lanes *1–6*) and supernatant fractions (lanes *1′-5′*). Two aliquots each were treated with protease, one of which received 0.2% Triton X-100 and 0.2% deoxycholate (*DOC*) as a control. Lanes *2″* and *5″* are the supernatants of the protease-treated fractions after reisolation of the organelles. All fractions were immunoblotted with antibodies against mouse DHFR, GAPDH, hsp60 and PGK (PGK-B). *Tet*, tetracycline; −/+, lysates of uninduced and constantly induced cells, respectively.

processed form (Fig. 10, *−chase*, lane *1*, *bottom*) and ~45% (38 ± 6.5%) in the unprocessed form (*top*). In the presence of aminopterin the same two products were observed (lane *4*). This time, however, ~75% (74 ± 6.3%) of the fusion protein was in the full-length form and only ~25% (26 ± 6.3%) in the processed form (Fig. 10, *−chase*, lane *4*). If after 3-h induction the cells were chased for another hour, ~61% of the protein was in the processed form in the absence of drug and ~50% in the presence of drug (Fig. 10, *+chase*, lanes *1* and *4*). (In the experiment shown the supernatant had some mitochondrial contamination, as shown by a faint trace of the mitochondrial marker hsp60 as well as a small leakage of fusion protein to the supernatants [*+chase*, lanes *1′–5′*].)

We already knew that in trypanosomes expressing Δb_2-DHFR, aminopterin was causing accumulation of both processed and unprocessed intermediates at the mitochondrial membrane. This was also likely to be true for Δb_2-DHFR-PGK-C, which meant that some of the unprocessed material would be in the glycosomes, and some stuck on the mitochondria. To distinguish these two classes of protein, cells expressing Δb_2-DHFR-PGK-C were induced for 3 h in the absence or presence of drug, fractionated with digitonin and the organellar pellet was run on a 0.6–2 M sucrose gradient. Virtually all the cytosolic content was in the post-digitonin supernatant (Fig. 11, *+/− aminopterin*, lane *s*), as indicated by the exclusive presence of PGK-B in this fraction (*crosses and dashed line*). The mitochondrial marker hsp60 (*circles*, *dotted line*) stayed mainly on top of the gradient, with the exception of a small second peak in fractions 7 (*−aminopterin*) and 6 and 7 (*+aminopterin*). The glycosomal marker GAPDH (*squares, continuous line*) peaks in fractions 7 (*−aminopterin*) and 6 and 7 (*+aminopterin*). In the absence of aminopterin, most of the DHFR fusion protein (*bars*) stayed on top of the gradient (lanes *1–3*), indicating its mitochondrial location. As expected, the proportions of the two bands resemble those seen in Fig. 10 (*−chase*, lane *1*), there being more processed form (*p*, *light shaded bars*) than unprocessed form (*black bars*). The glycosomal fractions contain a second peak of Δb_2-DHFR-PGK-C. This consists mainly of unprocessed protein (*black bars*) and a cleavage product (*p**) that migrates slightly slower than the true mitochondrial form. Given the relatively low level

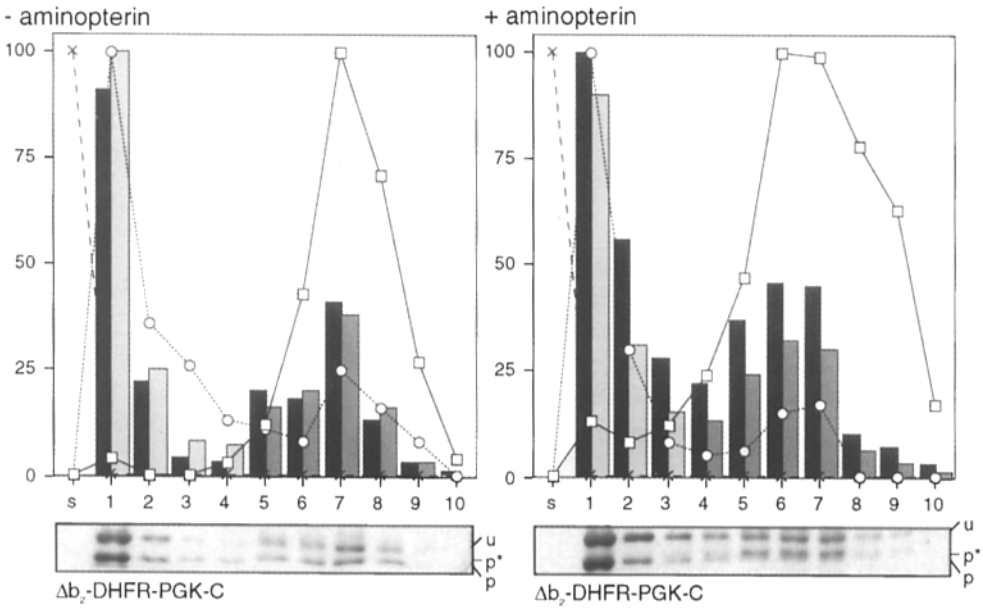

Figure 11. Sucrose gradient analysis of cells expressing Δb_2-DHFR-PGK-C grown in the presence (*+aminopterin*, *right*) or absence (*−aminopterin*, *left*). After 3 h of induction cells were harvested, fractionated as described and loaded onto an 0.6–2 M sucrose gradient. 10 fractions (fraction 1 *top*, fraction 10 *bottom*) were collected and analyzed by probing Western blots with anti-DHFR (*bars*), anti-GAPDH (□), anti-hsp60 (○), and anti-PGK (×) antibodies. For each antigen, values were normalized using the strongest band as a standard. The blot probed with anti-DHFR antibodies is shown below the graph. Processed (*p*, *light shaded bars*) and unprocessed (*u*, *black bars*) are indicated. Additionally an abnormally processed form (*p**) was detected in fractions 4–10. Darker shaded bars indicate the intensity of p and p* together, because accurate quantification of the closely apposed bands was not possible.

of mitochondrial marker in these fractions, most of this material is probably located in glycosomes. The abnormal cleaved product is present only in the glycosomal fraction; it could conceivably be generated by intra-glycosomal proteolysis. When aminopterin was included in the incubation, the relative distributions of the different forms changed. As seen before (Fig. 10, *−chase*, lane *4*) the unprocessed form became the predominant species, even in the mitochondrial fractions (Fig. 11, lanes *1–3*). In addition, there was a clear shift in the distribution of protein towards the glycosomal fractions. (Values are expressed relative to the strongest band, set arbitrarily at 100%.) The most conservative interpretation of all these data is that in the presence of aminopterin, the import of Δb_2-DHFR-PGK-C into mitochondria is inhibited, with build-up of precursor on the membrane, whereas import into glycosomes is either unaffected or even somewhat elevated.

As controls, we constructed cell lines expressing mutant Δb_2-DHFR-PGK-C or pcoxIV-DHFR-PGK-C unable to bind aminopterin (Vestweber and Schatz, 1988*b* and see above). As expected, addition of aminopterin had no effect whatsoever on the distribution between the processed and unprocessed forms (not shown). This corroborates the specificity of the aminopterin-induced effect on the wild-type fusion protein, and also confirms that the trypanosome processing protease is not affected by the drug.

These experiments demonstrated unequivocally that aminopterin could bind to Δb_2DHFR-PGK-C and pcoxIV-DHFR-PGK-C fusion proteins in vivo. As a consequence, the DHFR structure was stabilized sufficiently to reduce mitochondrial import, but import of the folded fusion protein into glycosomes was not inhibited.

Discussion

Aminopterin Does Not Inhibit Import of DHFR Fusion Proteins into Glycosomes

The transport of DHFR fusion proteins into yeast mitochondria in vitro is strongly inhibited by the addition of methotrexate (Eilers and Schatz, 1986; Rassow et al., 1989). The binding of the drug stabilizes the tertiary structure of DHFR, so that the protein can no longer unfold sufficiently to pass through the import channel. In vivo, import of such precursors into mitochondria is inhibited by aminopterin, a membrane-permeable version of methotrexate (Wienhues et al., 1991). The import channels are blocked with stuck intermediates, so that DHFR precursors (and other polypeptides destined for the same import pathway) accumulate outside the isolated organelle in vitro (Vestweber and Schatz, 1988*a*; Rassow et al., 1989) or in the cytoplasm and bound to the organelle in vivo (Wienhues et al., 1991).

When trypanosomes expressing a DHFR fusion protein targeted to glycosomes were incubated with aminopterin, we were unable to detect any significant inhibition of import by any criterion. The transport of newly synthesized protein was studied either by pulse labeling or by expressing the protein from an inducible promoter, and compartmentation was assessed by the only criteria available for glycosomes: cosedimentation with the organellar fraction, differential detergent permeabilization, protease-resistance, and immunoelectron microscopy. Although the conditions of the assay—aminopterin concentration, incubation and preincubation time, inclusion of sulfanilamide to deplete internal dihydrofolate pools (Wienhues et al., 1991)—were varied extensively, the only effect we observed was a delay in import that was too small to be statistically significant.

Aminopterin Can Enter Cells and Bind to the Targeted Fusion Protein

Given the unexpected nature of our results, it was very important to be sure that the DHFR fusion protein was actually binding the aminopterin in vivo. Control experiments confirmed that the levels of aminopterin used were in vast excess (100-fold) of those needed to kill both the original cells, and cells expressing the various hybrid proteins, unless thymidine was included to compensate for the inhibition of DHFR. Thus plenty of drug was entering the cells. Protease digestions also confirmed that the glycosomally targeted DHFR was able to bind drug in vitro, and that this binding stabilized the structure of the fusion protein sufficiently to inhibit protease digestion of the DHFR moiety. To prove unambiguously that the DHFR-PGK-C was actually binding the drug inside the cells, we devised control experiments employing fusion proteins targeted either to mitochondria or to both glycosomes and mitochondria.

Function of a Yeast Mitochondrial Targeting Signal in Trypanosomes

Since, to our knowledge, no mitochondrial targeting sequences had been functionally characterized in trypanosomes, we decided to test the well-characterized presequences of yeast cytochrome b_2 (van Loon et al., 1986; Beasley et al. 1993; Schwarz et al., 1993) and cytochrome oxidase subunit IV (Hurt et al., 1984, 1985). Analysis of import of both fusion proteins by biochemical and immunocytochemical methods convincingly showed that the heterologous targeting signals were functional in trypanosomes. This indicates that fundamental parts of the mitochondrial import machinery, including both the import receptor that recognizes the targeting signal and the mitochondrial processing protease, are conserved between the Kinetoplastidae and yeast.

Generation of a Mitochondrial Import Intermediate

Having established that the Δb_2 presequence was functional in targeting a protein to the trypanosome mitochondrion, we tested whether aminopterin induced formation of a mitochondrial import intermediate. Biochemical as well as immunoelectron microscopical evidence proved the existence of such intermediates. As was found in yeast, inhibition of import amounted to ~50%. In our experimental system, which required relatively long induction periods to allow detection and showed relative slow import and processing of the heterologous fusion protein, we detected both processed stuck intermediates and unprocessed fusion protein. Again, in yeast, after 3 h of induction ~70% of the protein accumulated as unprocessed forms (Wienhues et al., 1991).

In all experiments we found the stuck intermediates as-

sociated with the organellar fraction. This is in agreement with reports that similar fusion proteins with analogous internally deleted cytochrome b_2 presequences were retained by yeast mitochondria in vitro (Voos et al., 1993). In yeast, the retention was mediated by bound mt-hsp70; the dependency of this interaction on ATP was shown with other fusion proteins (Ungermann et al., 1994): only under ATP-depletion conditions could stuck fusion protein diffuse back out of the mitochondria. Since in vivo the mitochondria are presumably in an energized state, one would expect that stuck precursors would be retained, as we indeed found.

Behavior of Double-targeted DHFR Fusion Proteins

Having shown that the DHFR moiety binds its inhibitor in vivo, we tested the behavior of doubly targeted DHFR fusion proteins. The results of immunoelectron microscopy and cell fractionation showed that when doubly targeted proteins were expressed in trypanosomes, they were localized to both organelles. Western blots of steady-state cells revealed two principal DHFR species that could be assigned to the different organelles by sucrose density centrifugation: an unprocessed form in the glycosome and a shorter, processed form in the mitochondrion, besides a minor form that was cleaved by some proteolytic activity residing in glycosomes.

It is important to note that because of their location in the polypeptide, the mitochondrial presequence is synthesized first and the glycosomal targeting signal last, so that entry into mitochondria might be kinetically favored. If the protein were made on ribosomes that were actually bound to the mitochondrial outer membrane, the glycosomal targeting signal would be completely preempted by the mitochondrial one. Fujiki and Verner (1993) have indeed found that pcoxIV-DHFR is imported cotranslationally into yeast mitochondria in vivo. Thus it is not surprising that, under steady-state conditions, ~80% of the Δb_2-DHFR hybrid protein and 60% of the pcoxIV hybrid protein were found in trypanosome mitochondria. The relative numbers of the corresponding import receptors in the trypanosomes are also likely to play a role in the distribution. When production of the dual-targeting proteins was induced in the presence of saturating levels of aminopterin, much less of the protein was processed to the mature mitochondrial form. Sucrose gradient analysis showed the accumulation of stuck fusion protein associated with the mitochondria. As discussed above, this is probably due to the fact that the mitochondria actively retain the stuck constructs via bound mt-hsp70. The glycosomal import of the fusion protein was unaltered or slightly elevated by aminopterin under these conditions, but because the stuck precursor was retained by the mitochondria, no large redistribution towards glycosomes was observed. The behavior of a mutated, destabilized DHFR fusion protein was completely unaffected by aminopterin, supporting the specificity of the drug-induced effect on the wild type fusion protein.

We think that it is very important that all our experiments were conducted entirely in vivo, as the question of an in vitro artifact due to permeabilization or other organellar damage during isolation does not arise: the entire cell ultrastructure including all membranes is intact, and any cytoplasmic factors required (see below) are present at their normal concentration and location.

Is Unfolding Necessary for Glycosomal Import?

In comparison with mitochondrial studies, very little is known about the mechanism of peroxisomal or glycosomal import. However, what we do know is enough to tell us that the two import machineries are probably very different, and that what is true for glycosomes is probably true for other members of the microbody family. Whereas mitochondrial precursors are recognized by membrane receptors, there is evidence suggesting that initial recognition of some peroxisomal targeting signals occurs in the cytoplasm. Thus, Wendland et al. (1993) found that cytosolic factors were essential for import into peroxisomes in a permeabilized cell system, and presented evidence for the existence of SKL-binding sites in the cytoplasm; and PAS7, a protein involved in targeting of PTS2-containing proteins in *Saccharomyces cerevisiae* and a strong candidate for the PTS2 receptor, appears to shuttle between the cytoplasm and the peroxisomal membrane (Marzioch et al., 1994). In contrast to the import of mitochondrial precursors, import of peroxisomal proteins does not depend on the presence of a membrane potential (Imanaka et al., 1987). It is clear that import of at least some mitochondrial proteins depends upon their being kept in an "import-competent" state in the cytoplasm by cytoplasmic factors (Murakami and Mori, 1990; Sheffield et al., 1990), and that unfolding is necessary for import to occur, whereas for peroxisomal proteins that seems less likely to be the case. Import of prefolded proteins (including albumin decorated by multiple targeting peptides) into peroxisomes has been reported, either after microinjection into the cytoplasm or incubation with permeabilized cells (Walton et al., 1992*a*,*b*, 1994; Soto et al., 1993; Wendland and Subramani, 1993). Although the involvement of 70-kD heat shock proteins in peroxisomal import of prefolded polypeptides has been suggested, there is no evidence that these chaperones mediate unfolding of the imported polypeptides (Walton et al., 1994). Moreover, in vitro studies with the peroxisomal protein luciferase revealed that its folding is initiated cotranslationally (Frydman et al., 1994) and that the protein attains full enzymatic activity immediately after release from the ribosome (Kolb et al., 1994). These considerations by no means rule out the possibility that protein unfolding is necessary for import itself, but they also are clearly consistent with import in a (partly) folded state.

There are essentially two possible mechanisms whereby a folded DHFR fusion protein, stabilized by bound inhibitor, could be imported into glycosomes. One possibility is the presence of a strong unfolding activity present somewhere in the glycosomal import pathway. Such an activity would be able to remove aminopterin from the DHFR moiety, facilitating its uptake into the organelle. Such an unfolding activity has indeed been described for import of DHFR fusion proteins into chloroplasts (Guéra et al., 1993; America et al., 1994), but there is so far no evidence for an equivalent activity associated with glycosomes or peroxisomes. Although in vitro import of glycosomal phos-

phoglycerate kinase seemed to be stimulated upon urea denaturation of the precursor (Sommer et al., 1990), binding of precursors to glycosomes in the absence of import makes them less sensitive to protease, not more—the reverse of what would be expected if they were unfolded (Swinkels, 1989 and C. Clayton, unpublished data). The second possibility is that the proteins indeed enter in the folded state. This would imply either that the translocation channel is so large (or flexible) that it can accommodate large, folded complexes, or that some form of membrane internalization is involved (McNew and Goodman, 1994). There is convincing evidence for this option. Yeast peroxisomal thiolase can be imported as a dimer (Glover et al., 1994) and CAT-SKL even as trimer (McNew and Goodman, 1994); and most dramatically, Walton and colleagues (1995) could show that peroxisomes can import gold particles of a diameter up to 9 nm coupled to albumin-PTS1-conjugates. The existence of a flexible peroxisomal import channel is not inconceivable. Mitochondria have to sustain an electrochemical gradient across the inner membrane, so could not afford a large, "leaky" channel; indeed, accumulation of import intermediates in mitochondria does not lead to uncoupling (Wienhues et al., 1991). In contrast, although the glycosomal membrane seems to be rather impermeable to small compounds such as glycolytic intermediates (Hannaert and Michels, 1994), there is no evidence that it is impermeable to protons. This difference might allow glycosomes to employ an import machinery that is flexible enough to accommodate large and/or folded structures.

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