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Biogenesis of glycosomes of *Trypanosoma brucei*: An *in vitro* model of 3-phosphoglycerate kinase import

(*in vitro* transcription of cloned DNA/*in vitro* translation/proteinase K digestion/signal sequence)

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ABSTRACT **Glycosomes are intracellular, membrane-bound microbody organelles of trypanosomes and leishmania. Nine glycolytic enzymes are the major protein components of the glycosomes of *Trypanosoma brucei* long-slender bloodstream forms. Glycosomal proteins are believed to be synthesized in the cytoplasm and inserted across the glycosomal membrane posttranslationally. We have developed an *in vitro* protein import assay for the study of glycosomal biogenesis in *T. brucei*. All nine glycosomal glycolytic enzymes were detectable by immunoprecipitation and gel analysis of radiolabeled products derived from *in vitro* translation of total mRNA. Radiolabeled translational products were incubated with purified glycosomes isolated from bloodstream forms and digested with protease to remove proteins not imported into glycosomes. Gel analysis of reisolated glycosomes revealed that glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and 3-phosphoglycerate kinase (PGK) (EC 2.7.2.3) were apparently imported intact into the glycosome. Specificity of the protein import assay was verified by using translational products derived from cloned genes encoding *T. brucei* glycosomal PGK and its 95% homologous cytosolic isozyme. Glycosomal PGK was inserted into the glycosome *in vitro* with a 27.6% efficiency, but no imported cytosolic PGK was detectable. Preliminary data suggest that certain sequences between the N terminus and residue 123 may be important for import of glycosomal PGK. Our assay, combined with the potential use of genetically altered substrate proteins, may provide the opportunity to explore the recognition systems involved in glycosome biogenesis.**

African trypanosomes in the bloodstream of the infected mammalian host possess a significant metabolic deficiency—that is, their mitochondrion contains neither cytochromes nor a functional tricarboxylic acid cycle (1). Consequently, they are dependent entirely on glycolysis for energy. The rate of glycolysis in *Trypanosoma brucei* is 50 times higher than that in mammalian cells (2). This is apparently made possible by confining the first seven glycolytic enzymes and two glycerol-metabolizing enzymes within glycosomes (3), which are membrane-bound microbody-like organelles unique to parasites of the order Kinetoplastida. An understanding of the mechanism of biogenesis of glycosomes should be valuable in combating the diseases caused by these organisms.

T. brucei glycosomes contain 10% of the total cellular protein (4) but no nucleic acids (5). It has been assumed that the genes encoding glycosomal proteins (GPs) are located in the nucleus and the products of these genes are synthesized in the cytoplasm on free polysomes (6). Thus, glycosome assembly would require insertion of proteins across the organelle membrane posttranslationally. In the cases examined thus far, *in vitro* translation of mRNA yields GPs with the same molecular weights as the mature products inside the glycosome (6), suggesting that the import may not involve proteolytic processing. Thus, this process appears similar to the biogenesis of other microbodies, such as the peroxisomes of yeast and mammals (7–9) and the glyoxysomes of plants (10), and differs from the biogenesis of mitochondria and chloroplasts where posttranslational protein import generally involves proteolytic cleavage of specific leader sequences (11–13).

Studies of glycosome biogenesis in *T. brucei* have been hampered by the lack of an *in vitro* protein import assay (8), which has been instrumental to the biochemical analysis of protein transport into mitochondria (13) and chloroplasts (14). We have resolved the difficulties in preparing pure intact glycosomes with reproducible native membrane properties. Furthermore, the recent successful cloning and sequencing of several glycosomal genes (15–18) have enabled us to use gene clones to generate single gene products and to monitor their import into glycosomes individually. We report here the successful development of an *in vitro* GP import assay, which should allow dissection of the protein structural requirements for import.

MATERIALS AND METHODS

All chemicals used were obtained from Sigma, unless noted otherwise, and were of the highest purity available.

Growth and Isolation of *T. brucei* and Preparation of Glycosomes. The long-slender bloodstream form of *T. brucei* monomorphic strain EATRO 110 was grown and isolated as described (19).

For glycosome preparation, trypanosomes were suspended in an equal volume of 25 mM Tris·HCl, pH 7.8/1 mM EDTA/1 mM dithiothreitol/250 mM sucrose (TEDS) with 1 μM leupeptin and subjected to gentle homogenization by five strokes in a Dounce homogenizer (Kontes) with a small-clearance pestle (0.025–0.075 mm) at 4°C. Following centrifugation at 1000 × *g* for 10 min at 4°C, the pelleted nuclei and unbroken trypanosomes was resuspended and homogenized again as above. This procedure was repeated once more. All supernatants were pooled and centrifuged at 5000 × *g* for 10 min at 4°C to remove the large granular fraction. Glycosomes were pelleted by centrifugation at 33,000 × *g* for 30 min at 4°C and resuspended in TEDS at 1–2 mg of protein per ml. This suspension (3–4 ml) was layered on a 32-ml, 1–2 M linear sucrose gradient in TEDS and centrifuged at 49,000 rpm for 90 min at 4°C in a VTi-50 rotor in a Beckman L8-80M ultracentrifuge. The lower, prominent band was collected (ρ = 1.23 g/cm^3) (19), diluted

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Abbreviations: PGK, 3-phosphoglycerate kinase; g-, glycosomal; c-, cytosolic; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; GP, glycosomal protein.

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1:5 with TEDS, and pelleted by centrifugation at 33,000 × *g* for 30 min at 4°C. Purified glycosomes, resuspended in TEDS to a final concentration of 1 mg/ml, were stored at −70°C.

Antibody Preparation and Immunoprecipitation. Polyclonal antiserum to total GP (anti-GP) was raised in rabbits by using an initial intradermal injection of 0.75 mg of purified glycosomes in Freund's complete adjuvant and several booster injections at 2-week intervals with 0.75 mg of antigen in Freund's incomplete adjuvant. Reactivity to the variant surface glycoprotein was removed by absorption on a concanavalin A-agarose column previously saturated with the variant surface glycoprotein. Anti-GP was examined by immunoblotting experiments (20) and found to react specifically with the nine glycolytic enzymes in the purified glycosomes.

A 22-amino acid peptide, composed of an N-terminal cysteine plus the C-terminal 21 amino acids of glycosomal 3-phosphoglycerate kinase (EC 2.7.2.3; g-PGK), designated C-22, was synthesized by the Biomedical Resource Center, University of California, San Francisco, with an Applied Biosystem 430A peptide synthesizer and purified by C_{18} ODS reversed-phase HPLC. The final structure was confirmed by peptide sequencing: H_2N-Cys-Ser-Ala-Val-Val-Ser-Tyr-Ala-Ser-Ala-Gly-Thr-Gly-Thr-Leu-Ser-Asn-Arg-Trp-Ser-Ser-Leu-CO_2H (16). Antiserum to this peptide (anti-C-22) was prepared in a manner similar to that described above, except that initial immunizations of 1 mg, followed by 0.5-mg booster injections, were used. The N-terminal cysteine was originally added to the peptide to couple it to a carrier protein for the purpose of immunization. This was unnecessary as uncoupled C-22 was sufficiently immunogenic. Anti-C-22 reacts specifically with the g-PGK but will not recognize the cytoplasmic isozyme in immunoblotting experiments (20).

Indirect immunoprecipitation was performed on 1–3 μl of the *in vitro* translation mixture (see below) in 50 μl of 50 mM Tris·HCl, pH 7.8/150 mM NaCl/5 mM EDTA/1% (vol/vol) Triton X-100. Prior to use, protein A-agarose (Zymed Laboratories, Burlingame, CA) was washed three times with wash buffer (phosphate-buffered saline/10 mM methionine/1% Nonidet P-40/0.02% NaN_3) and resuspended in wash buffer with 1 mg of ovalbumin per ml. Samples were precleared with 10 μl (5 μg of protein) nonimmune rabbit serum and 20 μl of 50% (vol/vol) protein A-agarose for 1 hr at 4°C and centrifuged at 15,000 × *g* for 3 min at 4°C. The cleared supernatants were incubated at 4°C for 16–20 hr with 2–10 μl of antiserum (5 μg of protein) and then with 20 μl of 50% protein A-agarose for 1 hr. The complexes were collected by centrifugation as above and washed three times with wash buffer.

Preparation of *T. brucei* mRNA. Total RNA was extracted from long-slender bloodstream forms of *T. brucei*, purified by the guanidinium/cesium chloride method, and poly(A) RNA was selected by oligo(dT)-cellulose (Collaborative Research, Waltham, MA) chromatography (21).

Molecular Cloning and *in Vitro* Transcription of PGK Genes. By using the sequences of *T. brucei* strain 427 cytoplasmic PGK (c-PGK) and g-PGK (16) as a guide, two PGK-specific 20-mer oligonucleotides were synthesized. These were used to screen a genomic DNA library constructed of *T. brucei* DNA isolated from variant antigen type 1.7 of the IsTaR serodeme (22), kindly provided by Peter Myler (Seattle Biomedical Research Institute). Several clones were isolated and mapped by using restriction enzymes and oligonucleotide probes. Our maps of the PGK gene complex correspond closely to that described by Osinga *et al.* (16), with the exception of several restriction enzyme site polymorphisms. The identities of the g-PGK gene and c-PGK gene were determined by hybridization to isozyme-specific oligonucleotide probes, the position of the gene in the cluster, restriction enzyme mapping, and analysis of the protein products encoded by the gene.

The coding regions of the genes, plus a small amount of 5′ flanking sequences, were subcloned into pBS (Stratagene, San Diego, CA), a plasmid vector that contains the T3 and T7 promoters on either side of the polylinker cloning site. The c-PGK subclone, 123-2, contains 170 base pairs (bp) of 5′ noncoding sequence, whereas the g-PGK subclone, 44-1, contains 50 bp of the flanking region. In both cases, the first ATG codon starts the authentic protein. The plasmids were linearized, and transcripts were synthesized *in vitro* by using T3 or T7 RNA polymerase (Stratagene) according to the orientation of the gene.

***In Vitro* Translation and GP Import Assay.** RNAs were translated in a cell-free protein synthesizing system (nuclease-treated rabbit reticulocyte lysate, Promega Biotec, Madison, WI) containing 1 μCi (1 Ci = 37 GBq) of [^{35}S]methionine per μl (New England Nuclear, >800 Ci/mmol) for 60 min at 30°C. Typically 1 μg of RNA in a 50-μl reaction volume yielded 2–3 × 10^5 cpm/μl (for *T. brucei* mRNA) or 4–8 × 10^4 cpm/μl (for uncapped g-PGK or c-PGK gene transcripts) as trichloroacetic acid-precipitable protein. Immediately following this incubation, 2 M sucrose/25 mM Tris·HCl, pH 7.8/1 mM EDTA/1 mM dithiothreitol was added to yield a final concentration of 250 mM sucrose. Glycosomes at 0.4 mg of protein per ml in 240 μl of TEDS with 10 mM methionine/1 mM ATP/5 mM phospho*enol*pyruvate/2 mM $MgCl_2$/4 units of pyruvate kinase per ml were mixed with 10 μl of the translation mixture and incubated for 60 min at 37°C. Reaction mixtures were chilled at 4°C for 5 min, and proteinase K (Boehringer Mannheim) was added to 90 μg/ml. After 30 min at 4°C, proteolysis was terminated by the addition of 1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$), and the glycosomes were pelleted *in toto* at 15,000 × *g* for 15 min at 4°C. After one wash with 1 ml of TEDS/1 mM $PhMeSO_2F$, they were resuspended in $NaDodSO_4$ sample buffer with 1 mM $PhMeSO_2F$, heated at 95°C for 5 min, and stored at −20°C.

$NaDodSO_4$/PAGE and Fluorography. Samples were analyzed on 11% resolving gels with a 4% stacking gel by the method of Laemmli (23). ^{14}C-Methylated proteins (Amersham) were used as molecular mass standards. After staining with Coomassie brilliant blue, gels were treated with Enlightning fluorographic enhancer (New England Nuclear) and dried, and autoradiography was performed by using Kodak XAR-5 film at −70°C.

RESULTS

Development of an *in Vitro* Assay for the Import of Proteins into *T. brucei* Glycosomes. Lysis of the long-slender bloodstream forms of *T. brucei* in a Dounce homogenizer by a standardized gentle procedure freed glycosomes, which, after purification, resulted in homogeneous populations of intact glycosomes. These glycosomes consistently exhibited hexokinase (EC 2.7.1.1) latency (24) values of >75%, indicating a high level of membrane integrity. Latency was unchanged upon storage in TEDS at −70°C, facilitating assays of GP import. Native glycosomes of high latencies are essential for successful import assays. Fig. 1, lane A, presents the protein profile of such a typical preparation of glycosomes in $NaDodSO_4$/PAGE. The profile is remarkably reproducible; the identity of each major protein band has been repeatedly verified by previous purifications, activity assays, and characterizations (4, 19). The degree of homogeneity of the glycosome sample with such a typical protein profile has been demonstrated by electron microscopy (19).

An *in vitro* import assay, analogous to those used to assess protein import into mitochondria (25), chloroplasts (14), and

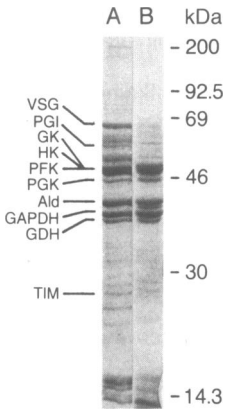

FIG. 1. $NaDodSO_4$/PAGE analysis of GPs before (lane A) and after (lane B) the *in vitro* protein import assay. VSG, variant surface glycoprotein; PGI, glucosephosphate isomerase; GK, glycerol kinase; HK, hexokinase; PFK, 6-phosphofructokinase; Ald, aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH, glycerol-3-phosphate dehydrogenase; TIM, triosephosphate isomerase.

peroxisomes (26, 27), was developed. This assay relied upon the presence of *in vitro* synthesized, radiolabeled polypeptides in the pellet fraction after a protease treatment known to digest all the proteins in the supernatant without damaging the glycosomal structure or contents. In our experience, trypsin was unsuitable since it either failed to digest all of the free proteins or began to digest the proteins originally inside the glycosome. Treatment with proteinase K at 90 μg/ml for 30 min at 4°C was suitable for eliminating most of the proteins from the supernatant fraction (see Fig. 4) without altering the GP profile in $NaDodSO_4$/PAGE (Fig. 1, lane B). External proteins associated with the purified glycosome, such as the 68-kDa variant surface glycoprotein (19) and the integral glycosomal membrane 24-kDa and 26-kDa proteins (28), were all digested by this protease treatment (Fig. 1, lane B).

***In Vitro* Import of GPs Translated from *T. brucei* mRNA.** Poly(A) RNA was isolated from long-slender bloodstream forms of *T. brucei* and used to program the synthesis of radiolabeled proteins in a rabbit reticulocyte cell-free translation system. By indirect immunoprecipitation with the antiserum to total GPs (anti-GP), polypeptides corresponding in size to the nine glycosomal enzymes were detected among the translational products in $NaDodSO_4$/PAGE (Fig. 2*A*). No larger precursors were observed, consistent with the previous observation that these polypeptides are synthesized at their mature sizes (6).

Inclusion of translational products of the total mRNA from the long-slender bloodstream *T. brucei* in the *in vitro* import assay resulted in the identification of several apparently imported proteins (Fig. 2*B*). The major protein bands at 47 kDa and 39 kDa and the minor bands at 41 kDa and 38 kDa correspond to the subunit molecular masses, respectively, of the glycosomal forms of PGK, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), aldolase (EC 4.1.2.13), and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8). The identity of g-PGK was verified by anti-C-22 immunoblotting experiments (data not shown). The other identifications must, however, remain tentative, especially for the minor protein bands since it is difficult to rule out the possibility of partial protease digestion of the imported major protein bands. By comparing the radioactivity corresponding to glyceraldehyde-3-phosphate dehydrogenase that is imported with that in the total translational products (data not shown), an import efficiency ranging from 4% to 6% was estimated. No radiolabeled proteins were detectable in control assays without glycosomes or with glycosomes lysed by $NaDodSO_4$ prior to proteinase K treatment (Fig. 2*B*) nor were there any radiolabeled proteins detectable in supernatant fractions in the absence or presence of glycosomes (data not shown). Glycosomes lysed by Triton X-100 were not included as a control because Clayton (29) has shown that when glycosomes are rendered permeable with nonionic detergents, GPs remain protease resistant. In the absence of added ATP and an

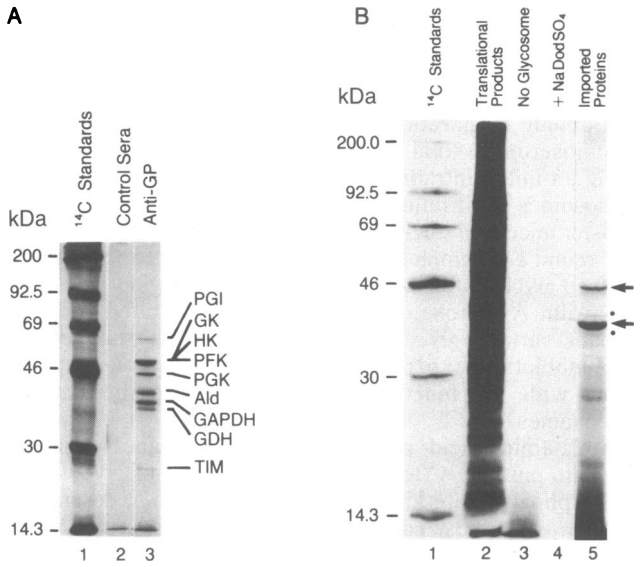

FIG. 2. (*A*) Identification of *T. brucei* mRNA translational products by indirect immunoprecipitation with anti-GP. Control serum was from rabbits prior to immunization. (*B*) *In vitro* import assay of *T. brucei* mRNA translational products. Lane 2, total translational products; lane 3, no glycosome control; lane 4, $NaDodSO_4$-treated glycosome control; lane 5, with glycosomes. Arrows indicate the major bands corresponding to PGK and GAPDH; dots indicate the minor bands corresponding to Ald and GDH. Both fluorographs (*A* and *B*) were exposed for 16 hr. See legend to Fig. 1 for abbreviations.

ATP-generating system, a much reduced protein import was observed. These results indicate that the assay can be useful for assessing the import of GPs *in vitro*.

Verification of the Specificity of the Glycosome *in Vitro* Protein Import Assay. If the imported 47-kDa band seen in Fig. 2*B* indeed represents g-PGK, then the protein synthesized from the cloned g-PGK gene transcript should also be imported. The specificity of the *in vitro* import system can be then investigated by analyzing import of the two PGK isozymes. The g-PGK and c-PGK genes subcloned into pBS were transcribed *in vitro* by using T3 and T7 RNA polymerases. These uncapped transcripts were then translated *in vitro*. Transcripts from the g-PGK gene clone directed the synthesis of a 47-kDa protein (Fig. 3*B*), as expected for g-PGK (16). Similarly, transcripts from the c-PGK clone yielded a 46-kDa protein (Fig. 3*B*), as expected for c-PGK (16). A series of smaller proteins was also observed in each case (Fig. 3*B*); their molecular masses were estimated to be 41, 34, 31.5, 29.5, and 26 kDa. On 10% gels, the 41-kDa band was resolved into a 39.5-kDa and 41-kDa doublet. These observed sizes correlate well with those of proteins predicted from the utilization of internal AUG codons as alternative initiation sites for protein synthesis. All of the gene products from both clones reacted with the antiserum to total GPs (anti-GP) (Fig. 3*A*), but only the g-PGK gene products, including all of the smaller polypeptides, reacted with the anti-C-22 antiserum directed against a peptide found only at the C terminus of g-PGK (Fig. 3*A*). Thus, the C-terminal region is present in all of the smaller polypeptides in the g-PGK translation mixture, consistent with each being a partial product of the g-PGK gene by internal initiation codon usage. A two-dimensional gel analysis of the g-PGK *in vitro* translational products also confirmed this hypothesis (M.P. and K. Alexander, unpublished observation).

When the g-PGK gene products were included in the glycosomal import assay, 27.6% of the total radiolabeled protein was found in the glycosome. $NaDodSO_4$/PAGE fluorographic analysis indicated the presence of three radio-

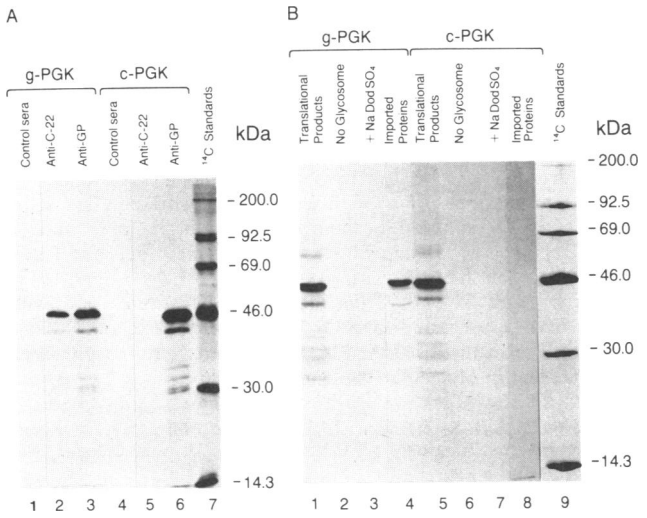

FIG. 3. (*A*) Indirect immunoprecipitation of gene products of clone 44-1 (g-PGK) and clone 123-2 (c-PGK) with anti-GP or anti-C-22. The fluorograph was exposed for 16 hr. (*B*) *In vitro* import assay using the same gene products. Lanes 1 and 5, total translational products; lanes 2 and 6, no glycosome control; lanes 3 and 7, NaDodSO$_4$/PAGE-treated glycosome control; lanes 4 and 8, with glycosomes. The fluorograph was exposed for 3 hr.

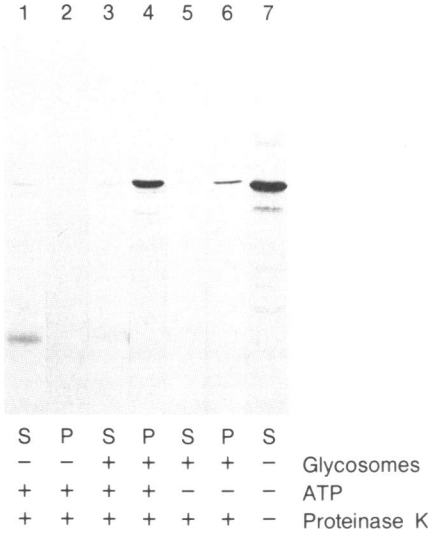

FIG. 4. Efficiency of proteinase K digestion of extraglycosomal proteins and the dependence of g-PGK import on added ATP in the *in vitro* assay. S, supernatant fraction; P, pellet fraction. Equivalent portions of supernatant and pellet fractions of each sample were analyzed. The fluorograph was exposed for 3 hr.

labeled bands in glycosomes: a 47-kDa species corresponding to the full-size g-PGK and two minor species of 41 kDa and 36 kDa (Fig. 3*B*). The exact nature of the latter two species is not clear. The 36-kDa protein in the glycosome is slightly larger than the 34-kDa protein in the *in vitro* translation of cloned g-PGK and has a distinctly different pI (M.P., unpublished results). Thus, its presence in the glycosome does not represent import of the 34-kDa protein. Whether the presence of a 41-kDa radiolabeled band in the glycosome represents import of one or both of the 39.5- to 41-kDa doublet species or whether it is a proteolytic degradation product of the 47-kDa protein remains to be elucidated. Similar experiments with equivalent amounts of c-PGK translational products resulted in no detectable radiolabeled proteins in the glycosome (Fig. 3*B*). The efficient, highly selective import of this well-defined GP, g-PGK, was conducted under the specific conditions that enabled proteinase K to degrade essentially all of the [^{35}S]methionine-labeled proteins in the supernatant fraction of the assay mixture in the absence or presence of glycosomes (see Fig. 4). No radiolabeled 47-kDa protein was detectable in the pellets of no glycosome controls, thus ruling out the possible formation of pelletable, proteinase K-resistant extraglycosomal 47-kDa protein during the assay (Fig. 4). The *in vitro* protein import was significantly decreased but not totally eliminated by not adding ATP, suggesting that the process could be ATP-dependent and that there could be residual ATP present in the assay mixture without further addition of ATP (Fig. 4). In all, we believe that the validity of this *in vitro* protein import assay has been established for the further study of glycosome biogenesis, though one cannot totally rule out at this time the possibility that g-PGK may bind specifically to glycosome and become resistant to proteinase K digestion.

DISCUSSION

One of the key obstacles to the study of protein transport into microbodies *in vitro* has been the lability of these organelles (8). The conventional method for the disruption of trypanosomes has involved the poorly controlled use of a harsh abrasive, silicon carbide (30), which results in intact glycosomes of variable membrane qualities not suitable for studies requiring native glycosomal membranes. We have established a reproducible method of breaking *T. brucei* to release the glycosomes, which, upon purification, yields intact glycosomes with relatively undamaged membrane. The conservation of >75% hexokinase latency in spite of freezing and thawing points to the suitability of these glycosomes for protein import assays.

ATP enhances the protein import, but the detailed mechanism of this ATP dependence is not yet clear. Little kinetic study has been performed, but preliminary observations suggested a very high rate of g-PGK import. Simultaneous additions of the precursor protein and proteinase K to the assay at 4°C allowed some import of g-PGK into the glycosome (unpublished results). Thus, some intact g-PGK may exist during the early phase of proteinase K treatment and limited import of g-PGK apparently occurs at 4°C. Though all nine [^{35}S]methionine-labeled glycosomal enzymes were present in the *in vitro* import assay, apparently only proteins corresponding in molecular mass to g-PGK and glyceraldehyde-3-phosphate dehydrogenase were imported efficiently. The failure among the other enzymes suggests that the optimal conditions for their import may differ. Further modifications of the import assay may be necessary for studying specific import of those proteins.

There are many indications that the results from our *in vitro* assays represent genuine g-PGK import into the glycosomes instead of g-PGK adsorption to the glycosomal membrane surface, which may somehow render the protein resistant to proteinase K. The step of proteinase K treatment in our assay, under the same condition known to remove the ADP/ATP carrier protein bound to mitochondrial membrane (31), can eliminate the nonspecifically bound variant surface glycoprotein (19) and the two integral membrane proteins (28) from the glycosomes. c-PGK, which has a 95% sequence homology on the nucleotide level with g-PGK but a lower pI value (16), is totally unprotected from proteinase K in the assay. The translational products of several recently constructed minor deletion mutants of g-PGK, exhibiting similar pI values as g-PGK in isoelectric focusing, were also tested in the assay; none was found protected from the proteinase K digestion (unpublished results). Since it is unlikely that nonspecific membrane adsorption of proteins should be affected at all by minor protein structural changes, the proteinase K susceptibility of c-PGK and the mutant

g-PGKs is more likely due to their inability to be transported into the glycosomes. This import process must thus be highly specific for g-PGK.

Recent studies on the genomic sequences of four *T. brucei* glycosomal enzymes, including g-PGK, indicated that none of them has a leader sequence (15–18), but all have remarkably high pI values (32). The high pI values are the result of basic amino acids interspersed along the molecules. Three-dimensional structural analyses of these enzymes performed by superimposing their deduced sequences on the crystalline structures of the same mammalian enzymes suggested that some of the extra basic amino acids are clustered in two areas on the surface of the molecule, 40 Å apart. It was postulated that these "hot spots" may be involved in the import of these proteins into the glycosome (33). The PGK isozymes offer an ideal system to dissect the structural requirements for glycosomal import: the relatively small (5%) sequence difference between g-PGK and c-PGK must play a role in differential localization. Aside from the hot spots found on g-PGK (but not c-PGK), other differences exist as well. For example, g-PGK possesses a unique C-terminal extension of 20 amino acids as well as numerous amino acid substitutions in the amino portion of the molecule. Another advantage that the PGK isozymes offer is that each is composed of a single polypeptide chain. Thus, the questions of import and conversion to protease resistance are not confounded by questions of subunit interactions.

Some insight concerning structural requirements for import may be gained by examining import of the smaller polypeptides generated by *in vitro* translation of g-PGK transcripts. These polypeptides react with an antiserum raised against the C-terminal 22 amino acids and have sizes and pI values corresponding to those predicted for C-terminal fragments of g-PGK synthesized as the result of internal methionine codon usage. Only full-size g-PGK (47 kDa) and possibly one or both of the 39.5- to 41-kDa doublet species (starting at the second methionine of the protein, amino acid residue 60) are imported (Fig. 3*B*). Polypeptides utilizing subsequent methionine codons as start codons are not imported. Since the fourth methionine occurs at amino acid residue 123 in the full-length protein, our data indicate that at least some sequences prior to amino acid residue 123 are important for import. This is where "hot spot I" resides. There are 18 other amino acid differences between g-PGK and c-PGK in this region. However, the possibility that only the 47-kDa protein was imported cannot be ruled out—that is, the two minor imported proteins of 41 kDa and 36 kDa could be derived from the 47-kDa protein by proteolysis in the glycosome or during glycosomal lysis. These findings will be investigated more rigorously with modified g-PGK gene products generated by deletion, insertion, and site-directed mutagenesis.

The primary sequence position of subcellular localization signals varies from protein to protein and organelle to organelle. Most mitochondrial localization signals (34, 35) are located at the N terminus. These signals usually have a relatively basic, amphipathic character that may facilitate their binding and entry into the mitochondrial membrane. An internal sequence in the large tumor antigen of simian virus 40 that is required for nuclear accumulation has been described; however, it is unclear whether this sequence represents a localization signal or whether it causes retention of the protein in the nucleus by interacting with other nuclear elements (36). C-terminal sequences required for nuclear localization of nucleoplasmin have been documented (37). Although import assays for peroxisomes (26, 27) and glyoxysomes (10) have been described, localization signals for peroxisomal or glyoxysomal proteins, presumably more closely related to the glycosomal signals, have not. However, the C-terminal half of *Candida tropicalis* acyl-CoA oxidase is correctly targeted in an *in vitro* peroxisomal uptake assay (38). Our data suggest that, at least in some cases, sequences in the N-terminal half of the molecule may be important for glycosomal localization.

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