Import of Fructose Bisphosphate Aldolase into the Glycosomes of *Trypanosoma brucei*

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Abstract. The glycolytic enzymes of Trypanosomatids are compartmentalized within peroxisome-like microbodies called glycosomes. Fructose bisphosphate aldolase is synthesized on free polysomes and imported into glycosomes within 5 min. Peptide mapping reveals no primary structural differences between the in vivo-synthesized protein and that made in vitro from a synthetic template. However, native aldolase from glycosomes is partially protease resistant, whereas the in vitro translation product is not. Pulse-chase results indicate that aldolase in bloodstream trypanosomes has a much longer half-life than in the procyclic tsetse fly form.

THE glycolytic enzymes of Trypanosomatids are compartmentalized in glycosomes, specialized microbodies that strongly resemble peroxisomes in morphology and physical properties (3, 31). Glycosomes are dense, spherical bodies with a diameter of 0.27 μm whose contents often appear aggregated to form a crystalline core. They are bounded by a single membrane and lack DNA (31). Although the compartmentalization of glycolytic enzymes is unique to Trypanosomatids, the glycosomes also contain other enzymes, such as those for ether lipid biosynthesis, which are present in the peroxisomes or glyoxysomes of higher eucaryotes (reviewed in reference 3). The shared properties suggest that all microbodies are evolutionarily related. Entry of proteins into peroxisomes and glyoxysomes is posttranslational. It differs from mitochondrial, chloroplast, and lysosomal assembly in that processing or maturation of component proteins is not obligatory for import (3, 22). It is therefore of evolutionary interest to determine whether glycosomes are constructed in the same fashion as other microbodies.

Substrate-level phosphorylation during glycolysis is the sole means of ATP regeneration in bloodstream trypanosomes (10), whereas the procyclic forms found in the tsetse vector have a fully active mitochondrion with oxidative phosphorylation. There is evidence that compartmentalization contributes to the very high rate of glycolysis in bloodstream forms (15, 27), and several parts of the glycolytic pathway have been exploited as targets for anti-trypanosomal drugs (10). A much slower glycolytic rate is required by procyclics, which have correspondingly reduced levels of some glycosomal enzymes: in particular, hexokinase and fructose bisphosphate aldolase (1, 17).

Hart et al. (16) have recently demonstrated that, in procyclic trypanosomes, several glycosomal enzymes are imported rapidly and posttranslationally, but are very unstable, having half-lives of only 30 min. This paper presents the in vivo characteristics of organellar import of fructose bisphosphate aldolase in bloodstream forms.

Materials and Methods

Trypanosomes and DNA Clones

Buffy coat trypanosomes (*T. brucei* strain 427, antigenic types 117, 118, or 221 [8]) were harvested from the blood of rats during the ascending phase of parasitaemia at parasite densities of ~5×10^8/ml. Aldolase and variant surface glycoprotein (VSG)[1] cDNAs have been previously described (5).

Polysomal RNA

Trypanosomes were harvested and purified in the presence of 10 μg/ml cycloheximide, then disrupted in a Stansted cell disrupter at 4,500 psi in polysome buffer (50 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 25 mM NaCl or KCl, 0.25 M sucrose, 10 μg/ml cycloheximide, 100 μg/ml heparin). All procedures were conducted at 4°C; the sucrose was essential to maintain RNA integrity. Nuclei and large debris were removed by centrifugation for 5 min, 2,000 *g*. The supernatant was then centrifuged at 30,000 *g* for 20 min, and the resulting pellet washed and resuspended in guanidinium thiocyanate to yield a membrane-bound RNA fraction. Detergent treatment for further membrane-bound polysome purification invariably resulted in RNA degradation. Free polysomes were isolated from the supernatant by pelleting through 0.5 and 1.0 M sucrose cushions at 200,000 *g* for 45 min (24). The RNA was extracted in guanidinium thiocyanate as before. Polysomal and total RNA were further purified by sedimentation through a cesium chloride cushion and analyzed by blot hybridization as described previously (5).

In Vivo Labeling

Bloodstream trypanosomes were washed at least three times in PBS containing 1% glucose and once in methionine-free medium. The medium used was DME supplemented with glutamine (0.3 g/liter), glucose (10 g/liter), Hepes (7.14 g/liter, pH 7.4), and either BSA (1 g/liter) or 5–10% dialyzed FCS. In some experiments 2-mercaptoethanol (5×10^{-6}M) was included. After a 15-min preincubation (37°C with gentle agitation at 2–5 × 10^7 trypano-

1. *Abbreviation used in this paper*: VSG, variant surface glycoprotein.

somes/ml), [^{35}S]methionine (7.5 μCi/ml at 800 Ci/m.mol) was added. In pulse-chase experiments, the chase of cold methionine gave a final concentration of 250 μg/ml (a 10^5-fold excess). The viability of the organisms was monitored microscopically throughout. Samples of 2–5 × 10^7 trypanosomes were taken and either ground for cell fractionation or lysed immediately in 25 mM Tris pH 7.5, 1 mM EDTA, 0.2% sodium deoxycholate, 0.2% Triton X-100, 50 μg/ml leupeptin.

Cell Fractionation

Trypanosomes were washed once in fractionation buffer (0.25–0.33 M sucrose in either 25 mM Tris [pH 7.5], 1 mM EDTA or 25 mM Hepes pH 7.4). In some experiments 2–5 μg/ml leupeptin was included. Organisms were resuspended in the same buffer (1 ml per 10^{10} trypanosomes) and ground with washed silicon carbide (1.5 g per 10^{10} parasites) for 2–2 1/2 min using either pestle and mortar or, for pulse-chase experiments requiring samples of 10^8 trypanosomes or less, a pellet pestle in 1.5-ml microfuge tubes. Silicon carbide was removed and washed by brief low speed centrifugation. A postnuclear supernatant was prepared by centrifugation at 2,000 *g* for 5 min and crude organelle pellets were produced by centrifugation at 48,000 *g* for 20–40 min. Clumped organelles could be dispersed using a pellet pestle. All procedures were performed at 4°C and were adapted from references 2 and 31. Between 50 and 80% of aldolase activity and protein was sedimented at 48,000 *g*. This variability suggests that aldolase in the supernatant was released from damaged organelles: small scale preparations appeared to have more glycosome leakage as disruption was less controlled. To assess glycosome integrity, aldolase was assayed by standard methods (23) except that 0.3 M sucrose was included in the assay buffer and 0.25 M sodium chloride was also added to counteract inhibition by endogenous RNA (27). Latent activity was revealed by addition of 0.1% Triton, which had no effect on the activity of externally added trypanosome aldolase. Aldolase remaining in the 48,000 *g* supernatant showed no latency but was in the partially protease-resistant form (see Results section), while latency of the organellar enzyme was variable but typically ~80% with a specific activity similar to that given in reference 27. The preparations were therefore comparable to those described previously (15, 27, 29).

Protease Treatment of Organelles

Total cell lysates, postnuclear supernatants, or organelle pellets resuspended in fractionation buffer were digested with 25–100 μg/ml of proteinase K, trypsin, or V8 protease. Each sample was derived from 2 × 10^7 trypanosomes resuspended in 20–200 μl fractionation buffer at protein concentrations of 20–200 μg/ml. Reactions were terminated with a cocktail of inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM *N*-tosyl-L-lysine chloromethyl ketone, 48 μg/ml aprotinin, 48 μg/ml soybean trypsin inhibitor, final concentrations) or with PMSF or soybean trypsin inhibitor alone as appropriate, and incubated for a further 15 min at 25°C to complete enzyme inhibition. Organelles were permeabilized with 0.1% or 0.2% Triton X-100 and in some experiments sodium chloride was added in a range of concentrations (0.1, 0.15, 0.25, and 0.5 M) to disrupt glycosomal enzyme aggregates (29, 30). The activity of the proteases was verified in all cases by examining the SDS-PAGE pattern of the total digest: VSG was susceptible to all the proteases used.

Immunoprecipitations

Fructose bisphosphate aldolase was purified from trypanosomes as described (26) and used for rabbit immunization. Preliminary titrations were performed to ascertain the amount of serum required for quantitative precipitation of aldolase from a given number of trypanosomes; preimmune serum was tested as negative control. The antiserum bound aldolase made in vivo or synthesized in vitro using either total trypanosome RNA or pure synthetic aldolase RNA as template. Organelles were permeabilized with 0.2% Triton X-100 in the presence of 2–50 μg/ml leupeptin then diluted at least 10-fold before precipitation. Immunoprecipitates bound to protein-A–Sepharose were subjected to high salt washes (170 and 500 mM NaCl) followed by a Tris-EDTA wash and analyzed by denaturing SDS-PAGE and fluorography. Accurate quantitation was achieved by excising the bands, solubilizing the gel in 80% Protosol/20% water (New England Nuclear, Boston, MA), and scintillation counting.

Peptide Mapping

^{35}S-labeled immunoprecipitated or in vitro–synthesized aldolase were denatured and run on 10% SDS polyacrylamide gels. Coomassie-stained bands were excised, loaded into the wells of a 15% polyacrylamide gel, and digested in situ with various proteases (7). The gel slices contained equivalent amounts of total protein as judged by Coomassie staining. The in vivo–labeled bands were pure aldolase, whereas the in vitro slices also included a variety of unlabeled reticulocyte proteins.

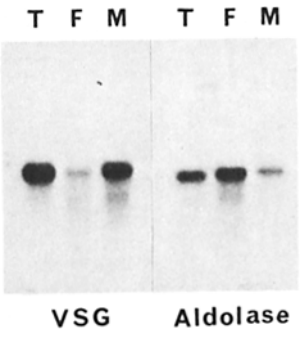

Figure 1. Aldolase is synthesized on free polysomes. Total (*T*), free polysomal (*F*), and membrane bound (*M*) RNA (5 μg each) were subjected to agarose gel electrophoresis, blotted onto APT paper (34), and hybridized with VSG or Aldolase probes.

Transcription and Translation

The full length aldolase cDNA (5) was subcloned into Gemini 4 (Promega Biotech, Madison, WI) and transcribed as suggested by the manufacturer except that the nucleotide concentrations were doubled to 1 mM. Tritiated nucleotides (Amersham Corp., Arlington Heights, IL) were included as tracers; the yield of RNA was 20–30 μg per 5 μg of input template. Synthetic and total or poly(A)$^+$ trypanosome RNA were translated in reticulocyte lysate or wheat germ cell free systems with an ATP regeneration system included (6). Reticulocyte lysate was obtained from Bethesda Research Laboratories (Gaithersburg, MD). Wheat germ extract was from Bethesda Research Laboratories or prepared as described (6). The potassium and magnesium concentrations were optimized for the capped and uncapped transcripts. Products were analyzed by SDS-PAGE and fluorography and TCA precipitation. Most translation of trypanosome sequences in the reticulocyte system ceased within 10 min, whereas that in wheat germ continued for up to 1 h.

Results

Polysomal Analysis

Membrane-bound and free polysomal RNAs were prepared from trypanosomes and analyzed by blot hybridization (Fig. 1) using aldolase and VSG probes. Aldolase RNA was highly enriched in the free polysomes (*F*), whereas most variant surface glycoprotein RNA was, as expected, in the membrane-associated fraction (*M*). This result implies that the import of aldolase into the glycosome occurs posttranslationally.

Peptide Mapping

As observed by Hart et al. (16) for procyclic trypanosomes, aldolase synthesized in vitro from either trypanosome mRNA or in vitro–synthesized message has a gel mobility indistinguishable from that of the in vivo–synthesized protein (not shown), suggesting that no covalent modifications that would affect gel mobility occur during import. This impression was confirmed by peptide mapping. The denatured proteins were digested with V8 protease and run on SDS-PAGE (Fig. 2). The maps of aldolase labeled in vivo (for 1 h) (lanes *1* and *3*) and during in vitro translation of a synthetic template (lanes *2* and *4*) are indistinguishable.

Protease Sensitivity

Before any experiments could be performed to analyze in

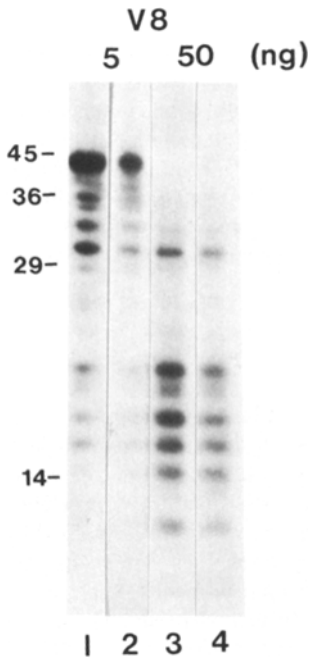

Figure 2. In vivo–labeled and in vitro–synthesized aldolase have identical peptide maps. In vitro–synthesized (2, 4) and in vivo–labeled immunoprecipitated (1, 3) aldolase were denatured and subjected to 10% SDS-PAGE. The aldolase bands were excised from the Coomassie-stained gel and inserted into wells of a 15% gel. 5 or 50 ng of V8 protease were then overlayed as indicated, the electrophoresis repeated, and the products visualized by fluorography. Molecular weight markers (kD) are indicated.

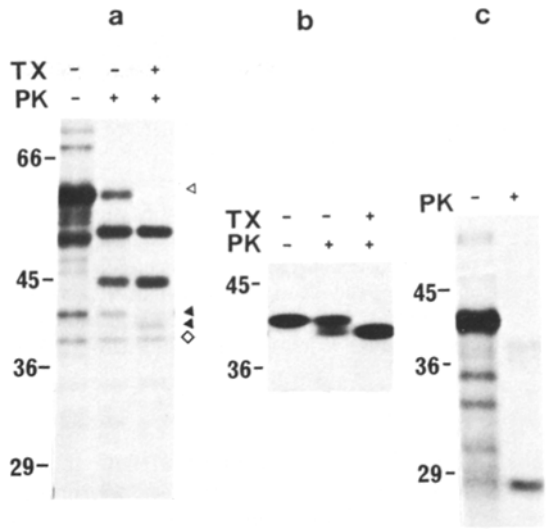

Figure 3. Digestion of aldolase with proteinase K. Silicon-carbide lysates from 2×10^7 in vivo–labeled trypanosomes resuspended in 200 μl fractionation buffer were digested with 50 μg proteinase K at 0°C for 30 min in the presence or absence of 0.1% Triton X-100. Samples were subjected to SDS-PAGE and fluorography. (*a*) One-tenth of the total digest; *open arrowhead*, VSG; *solid arrowheads*, aldolase and its digestion product. The band marked with a diamond has the same gel mobility as glyceraldehyde phosphate dehydrogenase. (*b*) Immunoprecipitated aldolase from the remaining sample as in *a*. (*c*) Aldolase made in reticulocyte lysate using a synthetic template, and subjected to SDS-PAGE with or without pretreatment with proteinase K (50 μg/ml, 0°C, 30 min). The faint protease product of molecular mass 38 kD is at least 1 kD smaller than the digestion product in *a* and *b*. Molecular mass markers (kD) are indicated.

vivo or in vitro glycosome assembly, it was necessary to find suitable conditions for proteolytic digestion of external components without compromising glycosome integrity. Crude parasite lysates, postnuclear supernatants or organelle pellets from in vivo–labeled trypanosomes were digested with varied amounts of proteinase K on ice or at 25°C for 30 min; the products were analyzed by SDS-PAGE and fluorography. Results of a typical experiment are illustrated in Fig. 3. The major labeled bands in a gel of total trypanosome lysate include VSG (*open arrowhead*) and glycosomal enzymes. A proteinase K concentration of 50 μg/ml at 0°C was sufficient to cleave most of the VSG to 52 kD (Fig. 3 *a*). Inclusion of 0.2% Triton X-100 completed the VSG digestion presumably because the remaining full-length VSG was released from vesicles (Fig. 3 *a*). In the absence of detergent some of the full length (41 kD) aldolase (Fig. 3 *a*, *solid arrowheads*, and Fig. 3 *b*) was digested by proteinase K to give a 39-kD product. The proportion digested varied from preparation to preparation: if the trypanosomes were processed rapidly with scrupulous avoidance of warming and in the presence of leupeptin (which does not inhibit proteinase K) almost no degradation was seen. When detergent was present, aldolase was quantitatively converted to the 39-kD form (Fig. 3, *a* and *b*). The extent of proteinase K cleavage seen in the absence of detergent was inversely correlated with enzyme latency and could therefore be used as a visual measure of organelle integrity. If trypanosomes were homogenized without leupeptin, the 39-kD aldolase degradation product was often seen in the absence of exogenously added protease; it was probably produced by endogenous protease activity (25). A band that co-migrated with glyceraldehyde 3-phosphate dehydrogenase was clearly visible in gels of Coomassie-stained (not shown) and in vivo–labeled organelle pellets (not shown) and total cell lysates (Fig. 3 *a*, *diamond*). This too was protease resistant.

Glycosomal proteins form aggregates within the organelle which can be dispersed by increasing the salt concentration to 150 mM (29). Trypanosome aldolase, like other class 1 aldolases, is a homotetramer in salt concentrations up to and including 0.5 M (27). To see whether the partial protease resistance of aldolase was a consequence of aggregation, digestions were performed in the presence of detergent and a range of sodium chloride concentrations at 4° and 25°C. The 39-kD product remained intact under all conditions tested (not shown). In contrast, in vitro–synthesized trypanosome aldolase was highly protease sensitive (Fig. 3 *c*); this result was confirmed in mixtures of the in vitro product and trypanosome organelle fractions or postnuclear supernatants (not shown). Even more dramatic results were obtained with trypsin (30 or 100 μg/ml), which digested in vitro–synthesized aldolase completely while leaving glycosomal aldolase in the same mixture completely untouched with or without detergent and salt. V8 protease (100 μg/ml) behaved similarly.

Kinetics of Aldolase Uptake and Turnover

The rate of aldolase entry into glycosomes was assessed by pulse-chase labeling and cell fractionation. The glycosomal location of aldolase was deduced either from its resistance to proteinase K digestion or by cosedimentation with the organelle fraction. As expected, the gel mobility of aldolase remained constant throughout the labeling period (Fig. 4). Even after pulses as short as 3 min, most of the labeled aldolase was either protease-protected or in the partially pro-

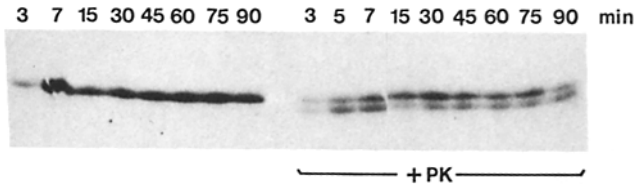

Figure 4. Kinetics of aldolase import. Trypanosomes were labeled with [^{35}S]methionine followed after 3 min by a cold chase. Samples of 5 × 10^7 organisms were taken at the times indicated and ground with silicon carbide. The resulting suspensions were treated with proteinase K (50 μg/ml, 30 min, 0°C) as indicated and quantitatively immunoprecipitated aldolase was subjected to SDS-PAGE.

tease-resistant form (Fig. 4) and sedimented with the organelle fraction (not shown). The exact rate of import could not be assessed because the time required for processing the labeled parasites was at least 3 min, so was greater than or equal to the half-time for uptake in vivo.

Hart et al. (16) found that in procyclic trypanosomes the glycolytic enzymes have half-lives of only 30 min. In contrast, I find that aldolase in bloodstream form trypanosomes is stable for at least 3 h after synthesis. This point is illustrated in Fig. 5. In this particular experiment, trypanosomes were labeled for 10 min before being chased (upper case lanes) or left for continuous labeling (lower case). Samples of the total lysate were taken and aldolase was immunoprecipitated. After autoradiography the immunoprecipitated aldolase bands were excised from the gel and counted. In the pulse-labeled culture the radioactivity associated with aldolase remained constant throughout the experiment. In gels of the total lysate, the glyceraldehyde phosphate dehydrogenase band also maintained constant intensity for the duration of the chase (not shown).

As bloodstream trypanosomes are much more difficult to maintain in vitro than procyclics, it was important to demonstrate that the parasites remained metabolically active throughout the culture period. The labeling medium had been optimized by inclusion of 2-mercaptoethanol (9) and (during the chase) complete FCS. The number of motile trypanosomes and the yield of protein (by Coomassie Blue stain of both total cell lysate and of immunoprecipitated aldolase) were constant throughout. This was expected as the division time in vivo is at least 8 h. The trypanosomes in the continuously labeled culture, which contained only dialyzed FCS and had very low levels of methionine, incorporated methionine into protein, including aldolase, in a linear fashion throughout the first 2 1/2 h (Fig. 5, *a–g*) by which time the level of radioactivity in the medium had halved. It therefore appears that in bloodstream trypanosomes aldolase and probably glyceraldehyde phosphate dehydrogenase are much more stable than in procyclics.

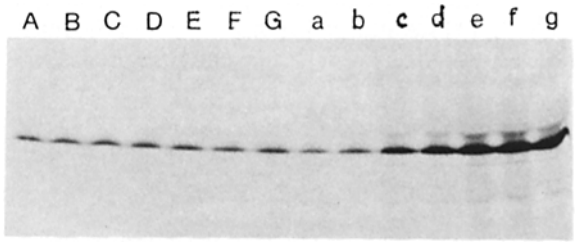

Figure 5. Glycosomal aldolase is stable in bloodstream forms. Trypanosomes were biosynthetically labeled with [^{35}S]methionine. Labeling was either continuous (lower case lanes) or for 10 min followed by a cold chase (upper case lanes). Samples of 2 × 10^7 trypanosomes were taken after: (*A*, *a*) 10 min; (*B*, *b*) 30 min; (*C*, *c*) 1 h; (*D*, *d*) 1 1/2 h; (*E*, *e*) 2 h; (*F*, *f*) 2 1/2 h; (*G*, *g*) 3 h. Aldolase was immunoprecipitated from detergent lysates and protein equivalent to 1 × 10^7 trypanosomes run on SDS-PAGE.

Discussion

Entry of fructose bisphosphate aldolase into the glycosomes of bloodstream trypanosomes closely resembles peroxisomal enzyme import. The protein is synthesized on free polysomes; subsequent glycosomal import occurs with a half-time of <5 min. These results agree with those obtained by Hart et al. (16) with procyclic trypanosomes. The posttranslational uptake of some peroxisomal enzymes, such as rat liver 3-ketoacyl Co-A thiolase (28), is also very rapid, but catalase takes 14 min (22) and yeast alcohol oxidase 20 min (14).

Posttranslational processing of peroxisomal enzymes does not appear to be obligatorily coupled to import. A minority undergo proteolytic cleavage but this maturation is slower than the import process (reviewed in reference 22). Oligomerization after import is however quite common. Kinetic studies indicate that catalase enters the peroxisome before it acquires a prosthetic heme group and forms an active tetramer (22). The cytoplasmic and in vitro–synthesized forms of cucumber malate synthase are monomeric and unable to oligomerize even when excess protein is added; the active octamer is found only inside the glyoxysomes (20, 21). In contrast, both glycollate oxidase (13) and catalase (22) can oligomerize in the cytoplasm. (Normal levels of active catalase can be detected in the cytoplasm of mutant human cells which lack peroxisomes [22].) A particularly relevant example is the alcohol oxidase of *Hansenula polymorpha*: the peroxisomal enzyme is a trypsin-resistant octamer, whereas the in vitro–synthesized monomer is trypsin-sensitive (33). The last result is reminiscent of trypanosome aldolase; peptide mapping of denatured aldolase reveals no structural alteration coupled to import but the native in vivo–synthesized form is markedly more protease-resistant than that made in vitro. The in vivo protein is untouched by trypsin and loses about 2 kD when treated with proteinase K, whereas the in vitro form is completely digested by both enzymes. Mature trypanosome aldolase, like the enzyme from higher eucaryotic sources, is a homotetramer (27), so it is possible that the partial protease resistance is consequent to oligomerization. The rabbit, chicken, and ascaris enzymes are also resistant to trypsin digestion (35) but the size of wheat germ aldolase is reduced by 1 kD by trypsin (35); this cleavage may be analogous to the proteinase K digestion of mature trypanosome aldolase. Several authors have suggested that oligomerization stabilizes the protomers of rabbit muscle aldolase (4, 18), but the situation is complicated by controversy over the degree of denaturation necessary to achieve complete dissociation (19). Aldolase partial-protease-resistance is attained at the same time as internalization within the glycosome; the resolution of the experiments was insufficient to distinguish

which happens first, but analogy with other systems suggests that the protein enters as a monomer.

There are several possible reasons why the in vitro translation product cannot attain the partially protease-resistant conformation. It may be too dilute to tetramerize in solution. Alternatively, it may be incorrectly folded or lack an essential covalent modification: for example, low levels of acetylation, methylation, glycosylation, or phosphorylation would not be detectable by peptide mapping. That could also explain why in vitro assembly experiments along the lines of those described by Fujiki and Lazarow (11) have so far failed (unpublished results), although the incubation conditions may have been unsuitable.

Wierenga et al. (36) have suggested that groupings of basic residues on the surfaces of glycosomal proteins may constitute import signals. This prediction is based on actual or predicted tertiary structures of the mature (and therefore presumably oligomerized) proteins (27). Its validity is dependent upon the assumption that the newly synthesized cytoplasmic and mature glycosomal enzyme conformations are similar in least with respect to the relative arrangement of the residues involved, a supposition that, for aldolase at least, clearly needs further examination in view of the differences in protease sensitivity. Another possibility, mentioned in reference 3, is that the overall basicity of glycosomal proteins may facilitate their retention within glycosomes. A reliable and efficient in vitro assembly system would greatly facilitate examination of these questions.

The most striking result to emerge from these studies is the stability of aldolase in bloodstream forms, which contrasts dramatically with its rapid decay (half-life of 30 min) observed by Hart et al. (16) in procyclics. The protein and enzyme activity (1, 17) are at least 30 times more abundant in the bloodstream forms, but the corresponding mRNA level is only sixfold higher (5). Differences in enzyme stability may well account for this anomaly. Hart et al. (16) argue that because the procyclic enzymes all have similar half-lives, each glycosome may turn over as a unit. This is a superficially attractive idea in view of the results presented here as isolated procyclic glycosomes are more fragile than the bloodstream organelles (1). However, it is difficult to reconcile with the very different degrees of regulation of the various glycosome components. Although aldolase is dramatically reduced in procyclics relative to bloodstream forms, glyceraldehyde phosphate dehydrogenase is decreased by only ~40% and the amount of malate dehydrogenase is increased at least 100-fold (1, 17). Extensive direct comparisons of several glycosomal enzymes in the two trypanosome types, under comparable culture conditions and using bloodstream forms that have adapted to continuous culture, will be needed to understand the contribution of glycosome stability to developmental regulation.

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