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Molecular Analysis of Glyceraldehyde-3-Phosphate Dehydrogenase in *Trypanoplasma borelli:* An Evolutionary Scenario of Subcellular Compartmentation in Kinetoplastida

Erik A.C. Wiemer,* Véronique Hannaert, Paul R.L.A. van den IJssel, Joris Van Roy, Fred R. Opperdoes, Paul A.M. Michels**

International Institute of Cellular and Molecular Pathology, Research Unit for Tropical Diseases, ICP/TROP 74.39, Avenue Hippocrate 74, B-1200 Brussels, Belgium

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Abstract. In *Trypanoplasma borelli,* a representative of the Bodonina within the Kinetoplastida, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was detected in both the cytosol and glycosomes. This situation is similar to that previously found in Trypanosomatidae, belonging to a different Kinetoplastida suborder. In Trypanosomatidae different isoenzymes, only distantly related, are responsible for the activity in the two cell compartments. In contrast, immunoblot analysis indicated that the GAPDH activity in cytosol and glycosomes of *T. borelli* should be attributed to identical or at least very similar proteins related to the glycosomal GAPDH of Trypanosomatidae. Moreover, only genes related to the glycosomal GAPDH genes of Trypanosomatidae could be detected. All attempts to identify a gene related to the one coding for the trypanosomatid cytosolic GAPDH remained unsuccessful. Two tandemly arranged genes were found which are 95% identical. The two encoded polypeptides differ in 17 residues. Their sequences are 72–77% identical to the glycosomal GAPDH of the other Kinetoplastida and share with them some characteristic features: an excess of positively charged residues, specific insertions, and a small carboxy-terminal extension containing the sequence -AKL. This tripeptide conforms to the consensus signal for targeting of proteins to glycosomes. One of the two gene copies has undergone some mutations at positions coding for highly conserved residues of the active site and the NAD^+-binding domain of GAPDH. Modeling of the protein's three-dimensional structure suggested that several of the substitutions compensate each other, retaining the functional coenzyme-binding capacity, although this binding may be less tight. The presented analysis of GAPDH in *T. borelli* gives further support to the assertion that one isoenzyme, the cytosolic one, was acquired by horizontal gene transfer during the evolution of the Kinetoplastida, in the lineage leading to the suborder Trypanosomatina (*Trypanosoma, Leishmania*), after the divergence from the Bodonina (*Trypanoplasma*). Furthermore, the data clearly suggest that the original GAPDH of the Kinetoplastida has been compartmentalized during evolution.

Key words: *Trypanoplasma borelli* — Kinetoplastida — Glyceraldehyde-3-phosphate dehydrogenase — Glycosome — Compartmentation — Isoenzymes

Nucleotide sequence data reported in this paper have been submitted to the EMBL/Genbank/DDBJ nucleotide sequence databases under accession number X74535

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); HK, hexokinase (EC 2.7.1.1); PGI, glucosephosphate isomerase (EC 5.3.1.9); PGK, phosphoglycerate kinase (EC 2.7.2.3); PYK, pyruvate kinase (EC 2.7.1.40); TIM, triosephosphate isomerase (EC 5.3.1.1); SDS, sodium dodecyl sulfate; SSC, saline sodium citrate (0.15 M NaCl, 15 mM sodium citrate, pH 7.0); MYR, millions of years

* *Present address:* Department of Biology, University of California at San Diego, CA, USA

** *Present address:* Department of Biochemistry, University of Nijmegen, The Netherlands

Correspondence to: P.A.M. Michels

Introduction

Trypanosoma spp. and *Leishmania* spp., protists belonging to the order Kinetoplastida, have been well studied mainly because they are responsible for a number of severe human disorders such as African sleeping sickness, Chagas' disease in South America, and different leishmaniases in all (sub)tropical regions of the world. A characteristic feature of the Kinetoplastida is the presence of glycosomes (Opperdoes and Borst 1977; Opperdoes 1987). These microbody-type organelles harbor enzymes involved in the β-oxidation of fatty acids and ether-lipid biosynthesis, purine and pyrimidine biosynthetic pathways, and glycerol metabolism (reviews: Opperdoes 1987; Hannaert and Michels 1994). Strikingly, seven glycolytic enzymes responsible for the conversion of glucose to 3-phosphoglycerate are localized in this organelle. This unique compartmentation of glycolysis has been the subject of extensive studies at both the molecular and enzymological level (Opperdoes 1987 and references therein). It has been argued that these organelles had, most likely, an endosymbiotic origin (Michels and Opperdoes 1991; Michels and Hannaert 1994). However, support for an endosymbiotic origin of glycosomes and other members of the microbody family is difficult to obtain; since these organelles do not contain DNA, all their proteins are encoded in the nucleus. Nevertheless, an analysis of glycolytic isoenzymes might provide a clue about the origin of the organelle. It was found that for two of the glycosomal enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Misset et al. 1987; Michels et al. 1991; Hannaert et al. 1992) and phosphoglycerate kinase (PGK) (Osinga et al. 1985; Swinkels et al. 1988) cytosolic counterparts exist.

For PGK, three clustered genes were identified, coding for one cytosolic and two glycosomal isoenzymes. These are highly similar and must have originated through gene duplication, followed by gene conversions partially erasing differences that arose by genetic drift (Le Blancq et al. 1988; Alexander and Parsons 1991; Swinkels et al. 1992). Therefore, PGK is not very informative concerning the organelle origin.

For GAPDH, the situation is quite different. Two tandemly linked genes could be detected that encode the glycosomal GAPDH in *T. brucei* (Michels et al. 1986), *T. cruzi* (Kendall et al. 1990), and *L. mexicana* (Hannaert et al. 1992). In addition, a single gene coding for a cytosolic GAPDH (Michels et al. 1991; Hannaert et al. 1992) was identified, localized elsewhere in the genome. Within each organism, the two genes for glycosomal GAPDH are completely identical. All glycosomal GAPDHs have several specific features—like an excess of positively charged amino acids, mostly localized in patches on the protein's surface (Wierenga et al. 1987), unique insertions, and a small C-terminal extension that presumably bears the glycosomal targeting signal (Blatner et al. 1992). Glycosomal GAPDH shares only 55% amino-acid identity with cytosolic GAPDH (Michels et al. 1991; Hannaert et al. 1992), which is 3.4 kDa smaller due to the absence of the insertions and C-terminal extension. Enzymological studies revealed unique kinetic features of the glycosomal GAPDH, most notably a reduced affinity for NAD^+ as a result of the peculiar structure of its coenzyme-binding pocket (Lambeir et al. 1991; Vellieux et al. 1993). Comparison of the amino-acid sequences of both isoenzymes and phylogenetic analysis suggested that the genes for the two GAPDHs are only distantly related and must have been acquired independently by an ancestor in the trypanosomatid lineage. It was therefore postulated that one of the GAPDH genes was obtained by horizontal gene transfer during evolution (Michels et al. 1991; Hannaert et al. 1992). Such a transfer must then have occurred before the divergence of *Trypanosoma* and *Leishmania,* estimated to be at least 400 MYR ago (Fernandez et al. 1993).

In order to investigate whether such gene transfer indeed took place, and if so, to determine which gene was involved, we examined the subcellular distribution of GAPDH in *Trypanoplasma borelli* and characterized its GAPDH genes. This organism parasitizes cyprinid fishes like carp, goldfish, and tench. *T. borelli* belongs to the suborder of the Bodonina, which forms the second main taxonomic group within the Kinetoplastida, along with the Trypanosomatina, to which, among others, *Trypanosoma* and *Leishmania* belong. Ribosomal RNA analysis by Fernandez et al. (1993) suggested that these lineages separated about 500 MYR ago. Opperdoes et al. (1988) have shown that the glycolytic pathway in *T. borelli* is compartmentalized in a similar way as in the Trypanosomatidae indicating that glycosomes were already present in the common ancestor.

In this study we report that GAPDH in *T. borelli* is also present in two cell compartments. However, only the enzyme related to the glycosomal GAPDH of Trypanosomatidae, and two highly similar genes encoding it, could be detected. No gene or gene product similar to the trypanosomatid cytosolic isoenzyme was found. The evolutionary implications of these findings are discussed.

Materials and Methods

Organisms. Strain K-100 of *T. borelli* (ATCC#50432) (Protista; order Kinetoplastida, suborder Bodonina) was originally isolated from blood of a naturally infected carp (*Cyprinus carpio*). This isolate was cultured axenically at 15–20°C in the biphasic blood-agar medium SNB-9 as described previously by Opperdoes et al. (1988) or in monophasic LIT medium containing, per liter water: 5 g liver infusion (Difco, USA), 5 g tryptose (Difco, USA), 8 g Na_2HPO_4, 4 g NaCl, 2 g D-glucose, and 0.4 g KCl. The pH was adjusted to 7.0. After filter sterilization, the medium was complemented with 10 mg sterile hemin (Sigma, USA) and heat-inactivated fetal bovine serum (Life Technologies, UK) to a final concentration of 10% (v/v). Routinely cells were harvested before or just after reaching their maximum cell density of 1×10^7 cells ml^{-1} and $5–6 \times 10^6$ cells ml^{-1} in blood agar and LIT medium, respectively.

Procyclic *T. brucei* (insect stage) cells, stock 427, were grown at

27°C in a semidefined synthetic medium (SDM-79) as described (Brun and Schönenberger 1979) and harvested in the late logarithmic phase of growth.

Digitonin Treatment of Intact Cells. Cells were harvested by centrifugation at 400*g* for 15 min at 10°C, washed twice with an isoosmotic buffer containing 25 mM HEPES (pH 7.4), 250 mM sucrose, and 1 mM EDTA, and finally suspended in the same solution containing 2 μg leupeptin ml^{-1}. Aliquots of the cell suspension were diluted in Hanks' balanced salt solution (1.3 mM $CaCl_2$, 5 mM KCl, 0.3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 138 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM $NaHPO_4$, and 15 mM HEPES pH 7.1). Digitonin, dissolved at increasing concentration in dimethylformamide, was added in a 1- to 20-μl volume to 300-μl cell suspensions containing 750 μg of protein. The mixture was incubated at 25°C for 4 min and then centrifuged for 2 min at 13,000*g*. Enzymes were assayed, immediately after centrifugation, in the supernatant. Total cellular enzyme activities were determined after addition of 0.1% (v/v) Triton X-100 to the cells.

Enzyme Determinations. Most glycolytic enzymes were assayed as described by Misset and Opperdoes (1984). Pyruvate kinase activity was measured as described by Callens et al. (1991). Protein concentrations were determined by the fluorescamine method (Stein et al. 1973) with bovine serum albumin as standard.

Gel Electrophoresis—Immunoblotting. Proteins in 20-μl aliquots of the supernatants of digitonin-treated cells were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE) according to Laemmli (1970), using a 8–18% polyacrylamide gradient gel. The size-fractionated proteins were electrophoretically transferred from the gel to an Immobilon PVDF Transfer Membrane (Millipore, USA). The membrane was saturated for 30 min in Tris-buffered saline (TBS) containing 5% low-fat milk and subsequently incubated overnight at 4°C in a 1/500 dilution of a polyclonal antiserum raised against purified *T. brucei* glycosomal GAPDH in the same buffer. The membrane was then washed two times in TBS containing 0.2% (v/v) Tween 20 and twice in TBS and incubated for 1 h in a 1/1,000 dilution of peroxidase-linked protein A. After three washes in TBS-Tween 20 and three washes in TBS, protein A–immune complexes were visualized by incubation in 50 ml TBS to which were added 10 ml of 4-chloro-1-naphtol (3 mg/ml) in methanol and 30 μl 30% H_2O_2.

Cloning and Sequencing of the GAPDH genes. From 4×10^8 *T. borelli* cells genomic DNA was isolated essentially as described for *T. brucei* by Van der Ploeg et al. (1982). A genomic library of *T. borelli* was constructed in *Escherichia coli* (strain MB406) using partially *Sau*3A-digested DNA, ligated into λGEM-11 (Promega, USA). Ligation to the phage arms, in vitro packaging, and infection of *E. coli* cells with phage particles were performed according to the directions of the manufacturer. The genomic library was screened for recombinant clones containing the gene for GAPDH by hybridization with a radiolabeled probe consisting of a 423-bp *Hin*dIII fragment of the *T. brucei* glycosomal GAPDH gene (Michels et al. 1986). Hybridization was performed at a stringency of 3 × SSC, 0.1% SDS, 5 × Denhardt's, in the presence of 25 μg ml^{-1} sheared herring sperm DNA and 10% (w/v) dextran-sulfate at 60°C. Posthybridization washes were carried out for 30 min at 60°C in 6 × SSC, 0.1% SDS; 5 × SSC, 0.1% SDS; and 3 × SSC, 0.1% SDS, respectively. Positive plaques on duplicate filters were selected and rescreened. High-titer phage lysates were prepared and the recombinant phage DNA was isolated. Restriction enzyme digests were made, size-fractionated on agarose gels, blotted to nylon membranes, and hybridized with the *T. brucei* glycosomal GAPDH probe using procedures described previously (Marchand et al. 1989). Two hybridizing *Eco*RI fragments (2.6 and 2.1 kb) and a *Sac*I fragment (0.6 kb) were subcloned in both orientations in pBSKS(+/–). In order to facilitate DNA sequencing a set of nested deletions was generated by exonuclease III (Erase-a-Base kit, Promega, USA). Plasmids with truncated inserts were transformed into *E. coli* XL-1 Blue. DNA sequencing of both strands was carried out according to the dideoxy chain-termination method of Sanger, using the T7 DNA polymerase sequencing kit (Promega, USA) with either single- or double-stranded DNA templates, purified as described (Marchand et al. 1989).

Three-dimensional Modeling. The modified amino-acid residues of the *T. borelli* GAPDH protein 1 were studied with the molecular modeling program Biograf 3.10 (Molecular Simulations Inc., USA) starting from the homologous structure of the *T. brucei* glycosomal GAPDH, as determined at a 3.2-Å resolution from Laue data by Vellieux et al. (1993). For point mutations, the complete protein was kept fixed; only the mutated side-chain was allowed to change its conformation (systematic search). For residues 91–94 the protein backbone was allowed to change its conformation, because the introduction of *T. borelli* Pro93 was not compatible with the ϕ/ψ backbone angles of *T. brucei* having Arg at the equivalent position. The backbone change posed no problems for the modeling as the residue preceding Pro is Gly.

Comparison and Alignment of Amino-acid Sequences of Glyceraldehyde-3-phosphate Dehydrogenases. Multiple alignment of amino-acid sequences was performed using the program Pileup of the GCG package (Wisconsin University, USA). The alignment was manually adjusted taking into account known elements of secondary structure as described by Fothergill-Gilmore and Michels (1993). Percentage of identity between pairwise aligned sequences was calculated with exclusion of areas containing deletions and insertions. The following amino-acid sequences were included: *Trypanoplasma borelli* glycosomal GAPDH 1 and 2 (this study); *Trypanosoma brucei* glycosomal and cytosolic GAPDH (Michels et al. 1986, 1991); *Leishmania mexicana* glycosomal and cytosolic GAPDH (Hannaert et al. 1992); *Trypanosoma cruzi* glycosomal GAPDH (Kendall et al. 1990); *Trichomonas vaginalis* (Markos et al. 1993); *Escherichia coli* GAPDH A and B (Branlant and Branlant 1985; Alefounder and Perham 1989); *Bacillus subtilis* (Viaene and Dhaese 1989); *Zymomonas mobilis* (Conway et al. 1987); *Rhodobacter sphaeroides* (Chen et al. 1991); *Aspergillus nidulans* (Punt et al. 1988); *Saccharomyces cerevisiae* (Holland and Holland 1979); *Zea mays* cytosolic GAPDH (Martinez et al. 1989); *Sinapsis alba* cytosolic GAPDH (Martin and Cerff 1986); *Arabidopsis thaliana* cytosolic GAPDH (Shih et al. 1991); *Schistosoma mansoni* (Goudot-Crozel et al. 1989); *Sus scrofa* (pig) (Harris and Perham 1968); *Gallus gallus* (chicken) (Stone et al. 1985); and *Homo sapiens* hepatic GAPDH (Tso et al. 1985).

Phylogenetic Analysis. Multiple alignments of amino-acid sequences were converted to the appropriate input format for the PHYLIP package (version 3.5 by Felsenstein 1993) using the program ReadSeq by Gilbert (1993). The number of substitutions between sequences was measured using the program PROTDIST (PHYLIP package) correcting for multiple substitutions according to Kimura (1983). Positions in which gaps were present in any of the aligned sequences were excluded from the analysis. Based on the distance matrix, a phylogenetic tree was inferred by the neighbor-joining method (Saitou and Nei 1987), implemented in the program NEIGHBOR (PHYLIP package). To test the reliability of the tree, the bootstrap method (Efron 1982; Felsenstein 1985) was applied. From the original set of sequences 100 bootstrap replicates were obtained for the construction of the corresponding distance matrices and phylogenetic trees. The program CONSENSE (PHYLIP package) was used to obtain a consensus tree as well as confidence levels for monophyletic groups. A parallel analysis was carried out using, in the phylogenetic tree reconstruction step, the least-squares method (Fitch and Margoliash 1967) as implemented in the program FITCH (PHYLIP package). Directly from the aligned amino-acid sequences a maximal parsimony analysis using the program PROTPARS (PHYLIP package) was made. A discussion on the reliability of the various tree-reconstruction methods can be found in

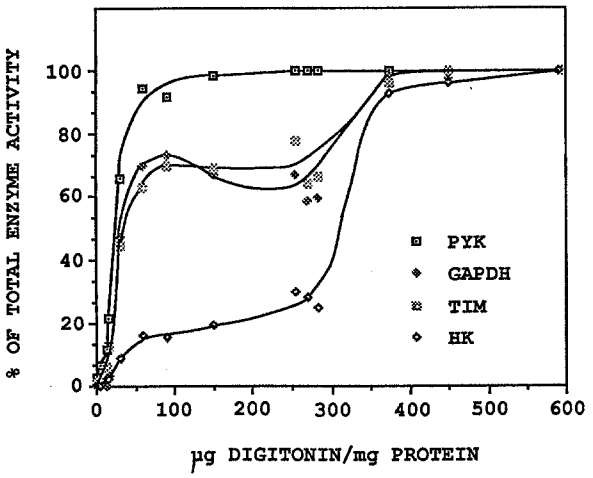

Fig. 1. Release of HK, TIM, GAPDH, and PYK from intact *T. borelli* cells by treatment with increasing concentrations of digitonin.

Hasegawa and Fujiwara (1993). All phylogenetic analyses were carried out on a R4000 Silicon Graphics Iris Indigo computer.

Materials. E. coli strain MB406 was obtained from Promega (USA), and *E. coli* XL1-Blue from Stratagene (USA). Restriction enzymes, nucleic-acid-modifying enzymes, and nucleotides were purchased from either Promega (USA) or Boehringer Mannheim (FRG) and used with buffers and under conditions as recommended by these companies. In vitro packaging of recombinant DNA in bacteriophage λ was performed with Packagene extracts (Promega, USA). The phagemid vector pTZ19R was purchased from Pharmacia LKB Biotechnology (Sweden), and the phagemid pBSKS (+/−) came from Stratagene (USA). Radiolabeled nucleotides [α-^{32}P]dATP and [^{35}S]dATP were obtained from Du Pont–New England Nuclear (USA). Nylon membranes (Hybond N) for blotting experiments and autoradiography films (Hyperfilm MP) were products of Amersham International PLC (UK).

Results

Subcellular Distribution of GAPDH in T. borelli

To determine the subcellular distribution of GAPDH in *T. borelli,* cells grown in the monophasic LIT medium were treated with increasing concentrations of digitonin. This detergent sequentially disrupts the integrity of the various membrane systems of a cell by forming an insoluble complex with their sterols. It has been shown that glycosomal membranes of Trypanosomatidae are rather resistant to digitonin (Visser and Opperdoes 1980; Hannaert et al. 1992). Figure 1 shows that all pyruvate kinase (PYK) was detectable at a low digitonin concentration (0.06 mg mg protein^{-1}), whereas the majority of hexokinase (HK) was only released at much higher concentration (0.37 mg mg protein^{-1}). About 70% of both GAPDH and triosephosphate isomerase (TIM) was detected in the supernatant after incubation at low digitonin concentrations, similar to PYK, whereas the remainder followed the activation pattern of HK. Similar results were repeatedly obtained using independently grown batches of cells. By fractionation of *T. borelli* homogenates, using differential centrifugation and isopycnic centrifugation in sucrose gradients, PYK was identified as a cytosolic enzyme, and HK was nearly completely found in the glycosomal fractions (Opperdoes et al. 1988; and unpublished data). Therefore, we conclude from the digitonin experiments that GAPDH is present in two cell compartments of *T. borelli,* as in Trypanosomatidae; about 30% of the activity is in glycosomes, 70% is in the cytosol.

Identification, Cloning, and Characterization of the T. borelli *GAPDH genes*

The genes encoding the glycosomal and cytosolic GAPDH of *T. brucei* were used to identify the GAPDH genes of *T. borelli.* Optimal hybridization conditions for library screening were established using Southern blots of genomic *T. borelli* DNA digested with various restriction enzymes. Specific hybridization with the glycosomal *T. brucei* gene probe could be observed at moderately stringent posthybridization washes: 1 × SSC, 60°C. However, no hybridization with discrete restriction fragments could be detected when the cytosolic GAPDH gene of *T. brucei* was used, even if the blots were washed at a stringency as low as 3 × SSC, 60°C. Only after long autoradiographic exposure (2 weeks) was weak hybridization observed with certain restriction fragments. However, these fragments appeared identical to those recognized by the glycosomal gene probe. Similar results were obtained with probes of *L. mexicana* cytosolic GAPDH and *E. coli* GAPDH-A. The latter gene has a high degree of similarity to the cytosolic GAPDH genes of Trypanosomatidae (Michels et al. 1991). These results strongly suggest that *T. borelli* does not contain a gene related to the cytosolic GAPDH gene of Trypanosomatidae.

A genomic library was constructed in λGEM11 and screened with the *T. brucei* glycosomal GAPDH probe at 3 × SSC, 60°C. Twenty positive clones were obtained. Five of them were used for gene mapping by Southern blot analysis and one for determination of the nucleotide sequence. Two GAPDH genes were detected, tandemly arranged as shown in Fig. 2. Both genes, the intergenic region, and part of the flanking areas were sequenced. The nucleotide sequence and the deduced amino-acid sequences are shown in Fig. 3.

Properties of T. borelli *GAPDH*

The GAPDH gene locus of *T. borelli* contains two open-reading frames coding for polypeptides of 362 amino acids with a calculated molecular mass of 39,063 and 39,222, respectively (excluding the initiator methionine). The two polypeptides differ in 17 residues. They are very

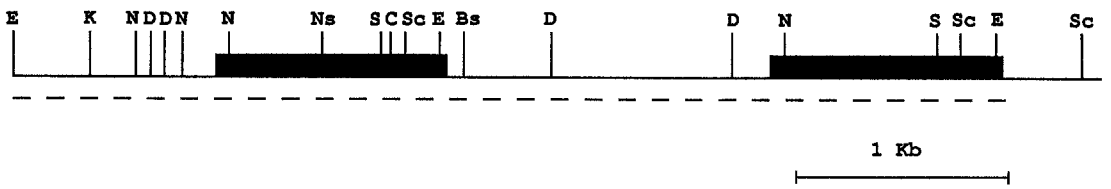

Fig. 2. Physical map of the GAPDH genes in *T. borelli.* The *solid blocks* indicate the protein-coding regions. The *broken lines* indicate the sequenced regions. Abbreviations: *Bs, Bst*EII; *C, Cla*I; *D, Dra*I; *E, Eco*RI; *K, Kpn*I; *N, Nde*I; *Ns, Nsi*I; *S, Sal*I; *Sc, Sac*I.

similar to, and clearly homologous with, the glycosomal GAPDH of Trypanosomatidae: the amino-acid sequences have 72–77% identity with the corresponding sequences of their counterparts in *T. brucei, T. cruzi,* and *L. mexicana.* The identity with the cytosolic GAPDHs of Trypanosomatidae, or with the enzymes of any other organism analyzed, is much less (≤57%). Moreover, the *T. borelli* GAPDH sequences share the characteristic features of the glycosomal GAPDH of other Kinetoplastida (Fig. 4). Specific insertions are present at the same position and all polypeptides have an excess of positively charged residues. The calculated net charge of the two *T. borelli* GAPDH polypeptides is +8 and +6; their estimated pI is 9.60 and 9.15, respectively. Furthermore, both polypeptides have a small carboxy-terminal extension containing the sequence -alanine-lysine-leucine (-AKL). This sequence is identical to that found at the terminus of *T. brucei* GAPDH. Using transfected *T. brucei* cells, it has been demonstrated that this tripeptide, fused to the C-terminus of a reporter gene, can function as targeting signal for glycosomes (Blattner et al. 1992; Sommer et al. 1992), as has previously also been shown for other microbodylike organelles (Gould et al. 1989).

The gene located downstream (designated gene 2) codes for a polypeptide that shares with the glycosomal GAPDH of Trypanosomatidae all residues which presumably are responsible for the 5–10-fold-reduced affinity of this enzyme for its coenzyme, as compared to their cytosolic isoenzyme and the GAPDH of all other organisms (Lambeir et al. 1991; Hannaert et al. 1992). These residues, specifically those closest to the adenosine moiety of NAD^+, appear to be more loosely packed around the cofactor (F. Vellieux, personal communication). However, the polypeptide encoded by the upstream gene (designated gene 1) has undergone some substitutions of generally conserved residues, particularly in the NAD^+-binding domain (Fig. 4, residues 1–166). Modeling of the protein's three-dimensional structure suggested that, nevertheless, none of these substitutions would dramatically affect the function of the enzyme. The effect of one major substitution, D38V, seems to be compensated by an additional change, Q49R, retaining a stable interaction with the ribose ring of the coenzyme. However, the binding of the coenzyme may be less tight. Modeling also suggested that the seemingly drastic substitution C170G of the noncatalytic cysteine would most likely not have a major impact. This is because a water molecule may be able to replace the cysteine side-chain. Moreover, substitution of this cysteine for serine has been reported for two thermophilic eubacterial sequences (Hecht et al. 1989; Schultes et al. 1990). Overexpression of the gene and kinetic analysis of the protein will be required to substantiate the theoretical considerations.

Phylogenetic Analysis

A phylogenetic tree was constructed based on aligned GAPDH amino-acid sequences and using the neighbor-joining method (Fig. 5). Essentially similar trees were obtained using least-squares and maximal-parsimony methods (not shown). The confidence intervals for the branching order in the tree were highly significant for the group of prokaryotic GAPDHs (including *T. vaginalis*), the group of glycosomal GAPDHs, and the group of trypanosomatid cytosolic GAPDHs (plus *E. coli* A). Thus the proposed evolutionary pattern is highly consistent. This pattern confirms the close relationship between the analyzed *T. borelli* GAPDH and the glycosomal isoenzyme of Trypanosomatidae. Together, they form a distinct branch that indicates an evolutionary early separation of the Kinetoplastida from the other eukaryotes. A similar conclusion has been reached previously, based on analysis of small-subunit rRNAs (Sogin et al. 1986), cytochrome c (McLaughlin and Dayhoff 1973), and other glycolytic enzymes (Michels and Opperdoes 1991). The tree in Fig. 5 also confirms our previous conclusion that the cytosolic GAPDHs of Trypanosomatidae form a separate group only distantly related to the glycosomal GAPDHs (Michels et al. 1991; Hannaert et al. 1992). The former group also comprises *E. coli* GAPDH-A. Nevertheless, this cluster is within the eukaryotic part of the tree, and all these sequences contain distinct eukaryotic features—for instance, in the so-called S-loop region (Branlant and Branlant 1985) (Fig. 4). A horizontal gene transfer has been invoked to explain the presence of a eukaryoticlike GAPDH in *E. coli* (Martin and Cerff 1986; Doolittle et al. 1990). However, Martin et al. (1993) postulated recently an alternative hypothesis—namely, that the nuclear gene for the glycolytic GAPDH in eukaryotes is of eubacterial origin. This hypothesis is based on their observation that also some cyanobacteria contain a eukaryoticlike GAPDH. The anomalous position of *Trichomonas vaginalis* among eubacteria in the GAPDH-tree has been discussed by Markos et al. (1993).

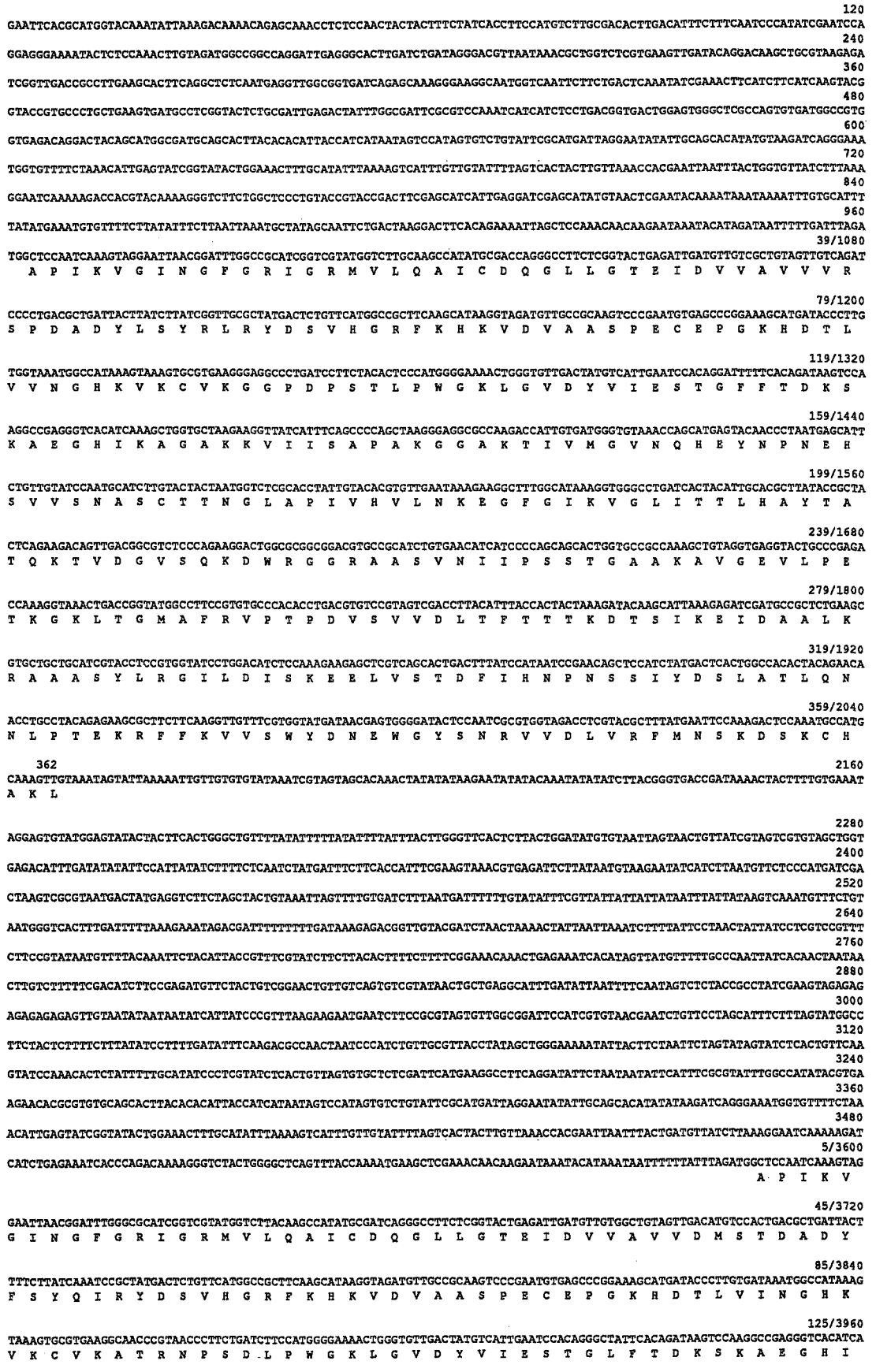

Fig. 3. The complete nucleotide sequence of the *T. borelli* GAPDH genes and their surrounding regions, and the deduced amino-acid sequences.

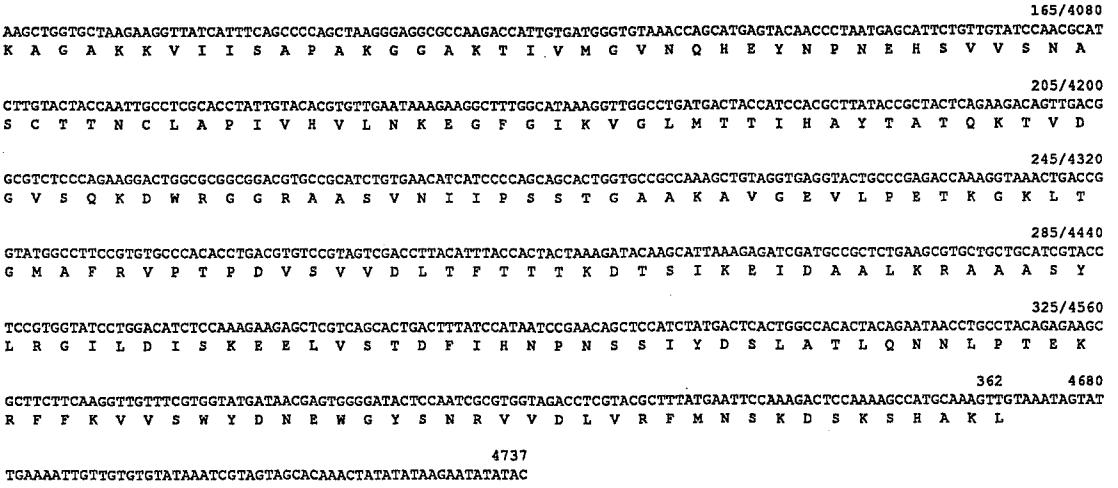

Fig. 3. Continued.

Identification of T. borelli *GAPDH by Western Blot Analysis*

The result of the digitonin titration experiments (Fig. 1) indicates that GAPDH is present in both the cytosol and in glycosomes of *T. borelli,* similar to our previous observations in various representatives of the Trypanosomatidae. However, only genes related to the glycosomal GAPDH gene of the trypanosomatids could be identified in the *T. borelli* genome (Figs. 2–5). All attempts to identify a gene related to the one coding for the *T. brucei* cytosolic isoenzyme remained unsuccessful. Therefore, we verified whether the products of the identified *T. borelli* genes would be responsible for the GAPDH activity measured in both compartments. Proteins released by incubation of *T. borelli* cells at different digitonin concentrations were size-fractionated by SDS/PAGE, blotted, and probed with an antiserum raised against *T. brucei* glycosomal GAPDH. In addition, a similar experiment was performed with procyclic *T. brucei* cells. Figure 6 shows that in the trypanosome the antiserum specifically recognizes the glycosomal GAPDH, as identified by its release at higher digitonin concentrations (Fig. 6, lanes 15–17). It does not cross-react with the highly different isoenzyme in the cytosol (Fig. 6., lanes 11–14). These results contrast with those obtained with *T. borelli,* where the antiserum reacts not only with the glycosomal GAPDH (Fig. 6, lanes 7–9) but also with the enzyme in the cytosol, which is detectable at lower detergent concentrations (Fig. 6, lanes 4–6). Moreover, the identity of the cytosolic and the glycosomal GAPDH in *T. borelli* is further supported by the observation that the protein recognized in the cytosolic fraction of *T. borelli* has the same subunit M_r as the glycosomal *T. brucei* GAPDH (approx. 39,000). This value corresponds well with the M_r predicted from the analyzed genes and is considerably larger than measured for the subunits of the cytosolic GAPDH (35,500) in Trypanosomatidae (Michels et al. 1991; Hannaert et al. 1992).

Discussion

Our results indicate that GAPDH is present in both the cytosol and the glycosome of *T. borelli,* as in Trypanosomatidae. In the latter organisms, the GAPDH activity in the two cell compartments can be attributed to highly different isoenzymes. In contrast, the cytosolic and organellar enzymes in *T. borelli* seem to be identical or at least very similar. They appear to be related to the glycosomal isoenzyme of Trypanosomatidae rather than to the cytosolic one, as judged from their antigenic properties and subunit M_r. The inference that *Trypanoplasma* does not have an isoenzyme related to the cytosolic GAPDH of Trypanosomatidae is supported by the results of our genomic analysis. No GAPDH gene related to that of the cytosolic GAPDH of *T. brucei* and *L. mexicana* could be detected; neither any evidence was obtained for the presence of other nonrelated GAPDH sequences.

From a comparison of the structure and organization of the GAPDH genes in Trypanosomatidae, and from a phylogenetic analysis, we previously hypothesized that it would be very likely that during evolution the Kinetoplastida had acquired the gene of one isoenzyme from a different organism by horizontal gene transfer. The studies in this paper strongly support this notion. The cytosolic GAPDH gene must have been acquired in the Trypanosomatidae lineage, after their separation from the Bodonina. The alternative hypothesis, that both genes were there before and that *Trypanoplasma* subsequently lost one of them, cannot be excluded. However, this possibility is considered less likely (Michels et al. 1991; Michels and Opperdoes 1991) and is not supported by some recent observations made on GAPDH in *Euglena gracilis.* (See below.)

We propose the following scenario for GAPDH gene evolution in Kinetoplastida. In ancestral Kinetoplastida, as in the present-day organism *T. borelli,* a single GAPDH was present, distributed over two cell compartments. Acquisition of a foreign gene enabled the Trypa-

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T.borelli 1      APIKVGINGFGRIGRMVLQAICDQGLLGTEIDVVAVVVRSPDADYLSYRLRYDSVHGRFK  60
T.borelli 2      ..............................DM.T....F..QI.........
T.brucei g        T..............F..L..D....N........DMNT..R.FA.QMK......K..
T.cruzi g         ...............F..L.ED............DMNT..E.FA.QM...T...K..
L.mexicana g      ...............F........I..........DM.TN.E.FA.QMKH.T....P.
T.brucei c        V.R..........V.FR.AQRRN    D.EI.GIND LL....MA.M.K...T....E
L.mexicana c       V...........V.FR.AQMRP    D.EI.GIND LL..E.MA.S.K...T....D
E.coli A          T............I.FR.AQKRS    D.EI..INDLL ....MA.M.K...T....D
B.subtilis        AV...........N.FR.ALNNP    .VE....NDLT ..NM.AHL.Q......KLD
Human            GKV...V........L.TR.AFNS.    KV.I..INDPFI.LN.MV.MFQ...T..K.H

T borelli 1      HKVDVAASPECEPGKHDTLVVNGHKVKCVKGGPDPSTLPWGKLGVDYVIESTGFFTDKSK  120
T.borelli 2      ...................I.........ATRN..D.............L......
T.brucei g       .S.STTK. KPSVA.D........RIL...AQRN.AD........E.......L..V..A
T.cruzi g        YE.TTTK. SPSVA.D........RIL...AQRN.AD........E.......L..A.AA
L.mexicana g     YT.EAVK. SPSVETA.V......RI....AQRN.AD..............L....L.
T.brucei c       GA.E.Q         GGA.....K KIR.TSER..AN.K.NEIN..V.V....L.LSDDT
L.mexicana c     GT.E.I         KGA.....K SIR.TSER..AN.K.DEI..EV.V....L.LTQET
E.coli A         GT.E.K         DGH.I...K KIR.TAER..AN.K.DEV...V.A.A..L.LTDET
B.subtilis       AE.S.D         GNN.....K TIE.SAER..AK.S...Q..EI.V.......KRAD
Human            GT.KAE         NGK..I..N PITIFQER...KIK..DA.AE..V....V..TME.

T.borelli 1      AEGHIKAGAKKVIISAPAKGGAKTIVMGVNQHEYNPNEHSVVSNASCTTNGLAPIVHVLN  180
T.borelli 2      ..................................................C.........
T.brucei g       ....LRG..R..V.....S.....F.....HNN...R.QH..........C...L....V
T.cruzi g        ....LRG..R..V.....S.....L.....H.....S..H..........C........V
L.mexicana g     ......G.....V.....S..............S.AS.H..........C........T
T.brucei c       .RK..Q......V.TG.S.DDTPMF.....HTT.KGE  AI.........C...LAK...
L.mexicana c     .HK..E...RR.VMTG.P.DDTPMF.....HTT.KGQ  PII........C...LAK.V.
E.coli A         .RK..T......VMTG.S.DNTPMF.K.A.FDK.AGQ  DI.........C...LAK.I.
B.subtilis       .AK.LE............NEEDI.......EDK.DAAN.D.I........C...FAK...
Human            .GA.LQG...R......SA D.PMF.....HEK.DNSL KII........C...LAK.IH

                                  S  LOOP
T.borelli 1      KEGFGIKVGLITTLHAYTATQKTVDGVSQKDWRGGRAASVNIIPSSTGAAKAVGEVLPET  240
T.borelli 2      ..........M..I.....................................................
T.brucei g       ......ST..M..V.S............V.........AL.....T........M.I.S.
T.cruzi g        .....VQT..M..I.S............V.........A......T........M.I.S.
L.mexicana g     ..N...ET..M..I.S............L.........A......T........KII.SL
T.brucei c       D K...VE..M..V..T.........P.........G.AQ..............K.Y.AL
L.mexicana c     E KY..VE..M..V..T.........P.L.......G..Q..........P....K....L
E.coli A         D N...IE..M..V..T.........P.H.......G..Q..............K....L
B.subtilis       D K....R.MM..V.S..ND.QIL.LP-H..Y.RA...AE....T........SL....L
Human            D N...VE..M..V..I.........P.G.L..D..G.LQ....A.........K.I..L

T.borelli 1      KGKLTGMAFRVPTPDVSVVDLTFTTTKDTSIKEIDAALKRAAASYLRGILDISKEELVST  300
T.borelli 2      ..................................................................
T.brucei g       Q............A.........IA.R..............SKT.MKN..GYTD.....A
T.cruzi g        Q......S.............AAR....Q.........SKT.MK...GYTD.....A
L.mexicana g     .......S.............RA.R....Q...K.I.K..QT.MK...GFTD.....A
T.brucei c       N............N.......VRLERPATY.Q.CD.I.A.SEGE.K...GYVD..I..S
L.mexicana c     D............N.......VRLE.PATY.D.C..I.A..EGEMK...GYTDD.V..S
E.coli A         N............N.......VRLE.AATYEQ.K..V.A..EGEMK.V.GYTEDDV...
B.subtilis       ....N.G.M.....N..L...VAELNQEVTAE.VN....E..EGD.K...GY.E.P...G
Human            N............AN.......CRLE.PAKYDD.KKVV.Q.SEGP.K...GYTEHQV..S

T.borelli 1      DFIHNPNSSIYDSLATLQNNLPTEKRFFKVVSWYDNEWGYSNRVVDLVRFMNSKDSKCHAKL 362
T.borelli 2      ..................................................S....
T.brucei g       ...SDSR......K........N.R....I..........H.......H.AAR.  RA...
T.cruzi g        ...NDNR......K........K.R....I..........H.......H.A...  RS.R.
L.mexicana g     ...NDNR..V...K........G..............A..H.......Y.AA.. AASS.M
T.brucei c       .INGI.LT.VF.AR.GISL.DN     .V.L.......T....K.H..IAHITK
L.mexicana c     ..NGVALT.VF.VK.GISL.DH     .V.L.......T...HK.L..ILHTSAR
E.coli A         ..NGEVCT.VF.GKGGMGL.DN     .V.L.......T....K.L..IAHISK
B.subtilis       .YNG.K...TI.A.S.MVMEGS     MV..I......S.........AAYIAK.GL
Human            ..NSDTH..TF.AG.GIAL.DH     .V.LI......F.........MAH.A..E
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Fig. 4. A comparison of the *T. borelli* GAPDH amino-acid sequences with the sequence of the corresponding protein from other organisms. *Dots* represent amino acids that are identical to the ones at the corresponding positions in the *T. borelli* glycosomal GAPDH (protein encoded by gene 1). *Blank spaces* indicate the absence of amino acids at corresponding positions. The S-loop sequence, which displays features diagnostic of either prokaryotes or eukaryotes, is labeled.

nosomatidae to develop isoenzymes specialized to their functioning in each of the compartments. The original enzyme became devoted to glycolysis in the glycosome; the newly acquired enzyme evolved to perform other functions (see below) in the cytosol. The feasibility of this scenario is supported by the observation that also in Trypanosomatidae such as *L. mexicana* several glycolytic enzymes (e.g., glucosephosphate isomerase [PGI]

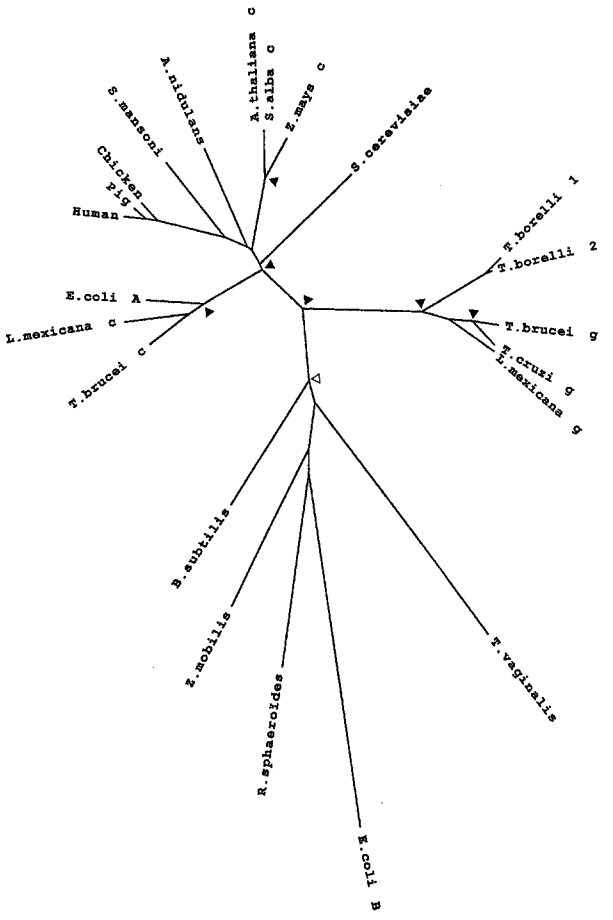

Fig. 5. Unrooted phylogenetic consensus tree inferred from GAPDH amino-acid sequences after 100 bootstrap replicates. The phylogenetic relationships were estimated by the neighbor-joining method. *Closed arrowheads at forks* indicate branching points that occurred in all 100 replicates. The branching point of the cluster of prokaryotic sequences (*open arrowhead*) occurred in 95 out of 100 times. For references to the methodology and the source of the sequences used for alignment and tree construction, see Materials and Methods.

and TIM) are present in two compartments (Hart and Opperdoes 1984; Kohl et al. 1994). Similar to GAPDH in *T. borelli,* no differences could be detected between the differently located enzymes, and only a single gene was found in the *L. mexicana* genome (Kohl et al. 1994; K. Nyame and P. Michels, unpublished results). It seems likely that the cytosolically located enzymes of *Leishmania* are involved in gluconeogenesis (Keegan and Blum 1993), and possibly in the pentose phosphate pathway, whereas the glycosomal enzymes perform glycolysis. This would prevent interference between the pathways, and energy loss by futile cycling. The usual kinetic mechanisms to prevent such interference (for a review, see Fothergill-Gilmore and Michels 1993) are not operational in Trypanosomatidae (Opperdoes 1987). This hypothesis is supported by the observation that in all Kinetoplastida analyzed, HK, an exclusively glycolytic enzyme, is only present in glycosomes. Moreover, in bloodstream-form *T. brucei,* where gluconeogenesis does not occur, the first seven enzymes (except GAPDH) of the pathway are (almost) completely found in glycosomes.

How a single enzyme would distribute over two cell compartments is not clear. Both *L. mexicana* PGI and *T. borelli* GAPDH have C-terminal tripeptides that have been shown very effective in targeting reporter proteins to glycosomes of *T. brucei* (Blattner et al. 1992; Sommer et al. 1992). Moreover the -AKL of *T. borelli* GAPDH is identical to the C-terminal sequence of the *T. brucei* enzyme that is only found in glycosomes. Partial targeting, as a result of an ineffective signal, seems therefore unlikely. Possibly, a part of the protein undergoes some minor modification, retaining it in the cytosol.

Two tandemly linked GAPDH genes were detected in *T. borelli,* as in all Trypanosomatidae. However, the two encoded *T. borelli* polypeptides are slightly different, contrary to the situation in *Trypanosoma* and *Leishmania.* The first polypeptide of *T. borelli* has undergone some unusual mutations, but modeling of its three-dimensional structure suggested that it could yet be functional. Both polypeptides contain the same putative glycosome targeting signal. There is, as yet, no indication that one polypeptide is specific for the cytosol and the other for glycosomes.

Our data do not provide new information about the origin of glycosomes and their early evolution. We consider a monophyletic, endosymbiotic origin of all microbodies, including glycosomes, most likely, based on arguments described previously by us and others (Borst and Swinkels 1989; Cavalier-Smith 1990; Michels and Opperdoes 1991; Michels and Hannaert 1994). However, our analysis of *T. borelli, L. mexicana,* and *T. brucei* GAPDH only allows the conclusion that an originally cytosolic enzyme can become fully compartmentalized during evolution. Further evidence for this assertion has been provided by studies on GAPDH of *E. gracilis,* a free-living photosynthetic protist. Morphological studies and rRNA analysis have shown that Euglenoida are more related to Kinetoplastida than any other protist (Sogin et al. 1986). Microbodies of *E. gracilis* do not contain any glycolytic enzymes (Opperdoes et al. 1988). Nevertheless, a GAPDH that is clearly homologous to the glycosomal isoenzyme of Trypanosomatidae was detected in this organism (W. Martin personal communication). This cytosolic *Euglena* enzyme shares all unique insertions of the trypanosomatid enzyme. However, it lacks the C-terminal extension with the microbody targeting signal. Furthermore, no GAPDH homologous to the cytosolic isoenzyme of trypanosomes was detected in *E. gracilis.* This provides further support for our hypothesis that the Trypanosomatidae acquired their cytosolic isoenzyme by horizontal gene transfer.

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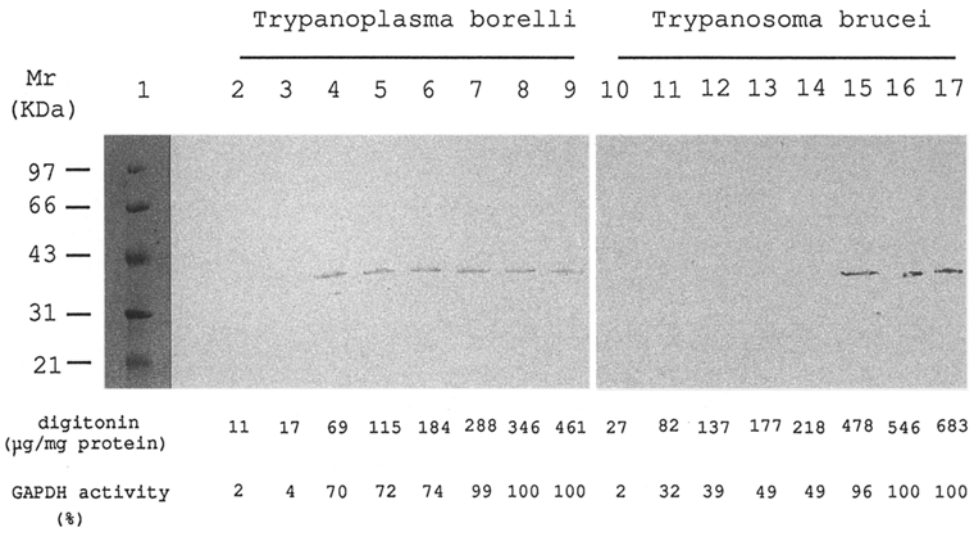

Fig. 6. Immunoblot analysis of soluble fractions of *T. borelli* (lanes 2–9) and procyclic *T. brucei* cells (lanes 10–17) after treatment with digitonin at increasing concentrations. The blot was probed with a polyclonal antiserum raised against *T. brucei* glycosomal GAPDH. The digitonin concentrations used and the released GAPDH activities are indicated.

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