

METABOLIC COMPARTMENTATION IN TRYPANOSOMES

Fred R. OPPERDOES, Member

Paul A.M. MICHELS, Member

Véronique HANNAERT, Assistant Member

Jean-Pierre SZIKORA, Assistant Member

Johan DE JONCKHERE, Visiting Scientist

Delphine GERBOD, Société Générale de

Belgique Fellow

Wilfredo QUINONES, Post-Doctoral Fellow

Marie-Astrid ALBERT, Graduate Student

Daniel GUERRA, Graduate Student

Murielle HERMAN, Graduate Student

Hanane KRAZY, Graduate Student

Juliette MOYERSOEN, Graduate Student

Cédric YERNAUX, Graduate Student

Freddy ABRASSART, Technician

Nathalie CHEVALIER, Technician

Dominique COTTEM, Technician

Christian VAN LANGENHOVE, Technician

Joris VAN ROY, Technician

Françoise MYLLE, Secretary



Trypanosomes are responsible for human sleeping sickness in tropical Africa and for a similar disease called 'nagana' in cattle. These are very serious diseases, with fatal outcome if left untreated. The presently available drugs are not very efficient and cause serious side effects. Moreover, development of drug resistant parasites is becoming a major problem. Therefore new drugs are badly needed.

Trypanosoma brucei, when it resides in the mammalian bloodstream, relies entirely on glycolysis for its ATP supply. Moreover, the parasite is characterized by a unique form of metabolic compartmentation; the majority of the enzymes of the glycolytic pathway is sequestered in peroxisome-like organelles called glycosomes (1). For the above reasons the glycolytic pathway is considered a validated and promising target for new drugs to be designed. Since many years we study the kinetic and structural properties of the glycolytic enzymes of T. brucei and closely related parasites such as Trypanosoma cruzi and Leishmania mexicana, and use the collected information for the design of effective and selective inhibitors by structure-based and catalytic mechanism-based approaches (2).

In addition our research aims at understanding what controls the glycolytic flux in vivo. The flux control is being studied in a quantitative manner by using a mathematical model prepared on the basis of the experimentally determined kinetic properties of all enzymes constituting the pathway, and by in vivo experiments in which the activity of different enzymes of the pathway is varied by either biochemical or genetic means. Such experiments could provide both insight into the consequences of the compartmentation of the pathway and information as to which enzymes of the pathway are the best targets for drugs.

Several enzymes of another pathway of carbohydrate metabolism: the hexose monophosphate pathway, involved in the generation of intermediates essential for cell growth, cell division and protection against oxidative stress, are associated with the glycosomes as well. This triggered our interest in their function as glycosomal proteins. Not only the role of the glycosome in trypanosomatid

metabolism is the topic of our research, but also the assembly of the organelle. We are studying the proteins, called peroxins, involved in glycosome biogenesis and particularly the mechanism by which they accomplish the import of matrix proteins.

Enzymes of carbohydrate metabolism

Cloning and characterization of genes for glycolytic enzymes, expression and characterization of recombinant enzymes and inhibitor development.

V. Hannaert, D. Guerra, M.-A. Albert, F. Oppendoes and P. Michels

In previous years, genes of all enzymes of the pathway have been cloned and characterized in our laboratory (3). In addition, we have cloned and sequenced the genes for two isoenzymes of the 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (PFK2/FBPase2), enzymes responsible for the synthesis and hydrolysis of fructose 2,6-bisphosphate, a potent allosteric effector of pyruvate kinase (PYK) and possibly the major regulator of glycolysis in trypanosomatids. Also genes for a number of glycolytic enzymes of *Leishmania* species and *T. cruzi* have been cloned and sequenced; in 2001 the genes for *L. mexicana* phosphoglycerate mutase (PGAM) and *L. donovani* phosphofructokinase (PFK) were characterized (4). The predicted primary structure of almost all trypanosomatid glycolytic enzymes appeared quite different from their mammalian counterparts and some enzymes are even not homologous, rendering several of these enzymes highly promising targets for anti-parasite drugs. Most of the *T. brucei* glycolytic enzymes have been expressed in *Escherichia coli* and purified and their kinetic properties have been determined.

In collaboration with C. López and J.L. Ramirez (Universidad Central de Venezuela, Caracas) the gene encoding *L. donovani* PFK was cloned and sequenced and its bacterially expressed gene product studied (4). The deduced polypeptide contains a C-terminal type I peroxisome-targeting signal (PTS1), -SKV. Like that of the previously characterized *T. brucei* PFK (70 % identity), the sequence showed the highest similarity to inorganic pyrophosphate (PP_i)-dependent PFKs, despite being ATP-dependent. Its kinetic properties were similar to those of the *T. brucei* enzyme. Modeling studies and site-directed mutagenesis were employed to shed light on the structural basis for the unique AMP effector specificity and on ATP/PP_i specificity among PFKs.

The predicted amino-acid sequence of *L. mexicana* PGAM is 74 % identical to that of the *T. brucei* mutase. Both trypanosomatid PGAMs belong to the class of cofactor 2,3-bisphosphoglycerate independent mutases, contrary to the non-homologous cofactor-dependent enzyme of humans. The parasite enzymes have been expressed in *E. coli*, and, upon purification, their kinetic properties have been determined.

During previous years, Prof. W. Hol (University of Washington, Seattle, USA) and Dr. L. Gilmore (University of Edinburgh, UK) and their coworkers have, in collaboration with us, established the crystal structure of several trypanosomatid glycolytic enzymes: aldolase (ALD), triosephosphate isomerase (TIM), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-phosphoglycerate kinase (PGK), glycerol-3-phosphate dehydrogenase (GPDH) and pyruvate kinase (PYK). Based on these crystal structures, and on insight in the kinetic properties and catalytic mechanisms, inhibitors of each of these enzymes have been designed and synthesised by our colleagues Prof. J. Périé (Université Paul Sabatier, Toulouse, France) and Prof. M. Gelb (University of Washington, Seattle, USA). Promising inhibitors, selective for parasite GAPDH and ALD, with K_i's in the nanomolar range and inhibiting growth of parasites in culture without affecting growth of cultured human cells have already been obtained.

In collaboration with Dr. M. Willson and Prof. J. Périé (Université Paul Sabatier) we also synthesized a number of glucosamine analogues which inhibit both the yeast and the *T. brucei* hexokinase (HXK) (5). The most potent inhibitor of the trypanosome HXK, *m*-bromophenyl glucosamide, did not affect the activity of its yeast counterpart. This compound also effectively inhibited growth of *in vitro* cultured trypanosomes. The structure of the *T. brucei* enzyme, in complex with its *m*-bromophenyl glucosamide inhibitor, was modelled using the crystal structure of the *Schistosoma mansoni* HXK - glucose complex. This model allowed us to explain the mode of action of this inhibitor on the trypanosome HXK.

Also in collaboration with our colleagues in Toulouse, the kinetic mechanism of *T. brucei* PFK has been studied in detail, and its active site has been explored by using a variety of inhibitors derived from the fructose 6-phosphate analogue 2,5-anhydromannitol (6). The best inhibitor was a compound with an electrophilic isothiocyanate

group at position 1; it displayed an irreversible inactivation pattern with a K_i value of 133 μ M. The residue involved in the specific inactivation of the parasite enzyme was identified by site-directed mutagenesis, Lys227. Based on this promising result, other compounds are now being developed.

The cloning of the *T. brucei* enolase (ENO) gene was reported last year. The encoded polypeptide has 59 – 62 % identity with the different human enolases.

The kinetic properties of bacterially expressed *T. brucei* ENO are very similar to those of the mammalian enzymes. Furthermore, structure modelling (in collaboration with Dr. D. Rigden, (CENARGEN/EMPRAPA, Brasilia, Brazil) indicated that the overall conformation of the active site of the trypanosomal enzyme is very similar to those of the enzyme from yeast and lobster for which crystal structures are available. However, there are some atypical residues (one Lys and two Cys residues) close to the *T. brucei* active site. These residues could possibly be exploited for the irreversible binding of selective inhibitors. The accessibility of these residues for inhibitors is currently being studied in collaboration with Dr. D. Vertommen (HORM Unit).

The activity of trypanosomatid PYK is allosterically regulated by fructose 2,6-bisphosphate (F-2,6-P₂), contrary to the PYKs from other eukaryotes that are usually stimulated by fructose 1,6-bisphosphate (F-1,6-P₂). The molecular basis of the specificity for the allosteric effector was studied in more detail in collaboration with Drs. D. Rigden (Brasilia) and L. Gilmore (Edinburgh)(7). Based on the comparison of the three-dimensional structure of *Saccharomyces cerevisiae* PYK crystallized with F-1,6-P₂ present at its effector site (R-state) and the *L. mexicana* enzyme crystallized in the T-state, two residues (Lys453 and His480) were proposed to bind the 2-phospho group of the effector. This hypothesis was tested by site-directed mutagenesis. The allosteric activation by F-2,6-P₂ appeared to be entirely abrogated in the mutated enzymes confirming our predictions. In addition, we have prepared two mutants for use as tools to screen the large number of compounds we anticipate from ligand docking, database mining and combinatorial chemistry. Wild-type trypanosomatid PYK has no tryptophan residues, and we have introduced this residue into two different positions near the effector site (F442W and E451W). Both mutants show fluorescence quenching in response to substrates and effectors, and will thus play an important role in screening combinatorial libraries.

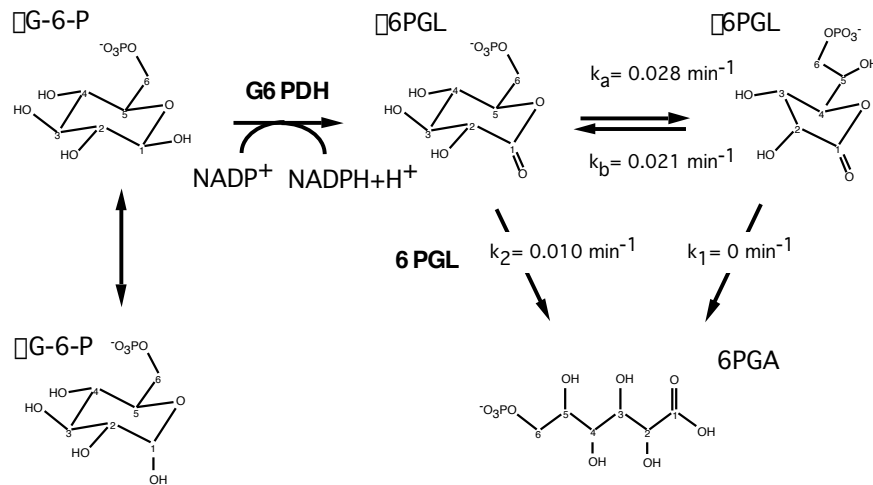


Fig. 1. Proposed scheme for the first two steps of the HMP. Glucose 6-phosphate (G-6-P) oxidation by G6PDH leads to the formation of α -6-phosphogluconolactone (α -6-P-G-L). The two 6-P-G-Ls are in exchange, characterized by k_a , the rate constant of the conversion of α -6-P-G-L into β -6-P-G-L, and k_b , for the reverse reaction. No spontaneous hydrolysis of α -6-P-G-L was measured.

Enzymes of the hexose monophosphate pathway (HMP)

Glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase

E. Saavedra, P. Michels and F. Opperdoes, in collaboration with F. Duffieux, Université de Paris Sud, France

Previously, we reported the cloning and characterization of *T. brucei* glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconolactonase (6PGL). We have now also cloned, characterized and expressed the corresponding genes of *L. mexicana*.

In collaboration with Dr. Duffieux (Paris) the three-dimensional structure of the *T. brucei* 6PGL is being solved using the technique of nuclear magnetic resonance. So far, the role of this enzyme in metabolism was still questionable, because 6-phosphogluconolactones were believed to undergo rapid spontaneous hydrolysis. By using both ^{13}C and ^{31}P -nuclear magnetic resonance spectroscopy we have characterized the chemical scheme and kinetic features of the oxidative branch of this pathway (Fig. 1) (8). The \square form of the lactone is the only product of glucose 6-phosphate oxidation. It leads to the spontaneous formation of the \square form by intramolecular rearrangement. However, only the \square form undergoes spontaneous hydrolysis, the \square form being a "dead end" of this branch. The \square form is the only substrate for 6PGL. Therefore, the activity of this enzyme accelerates hydrolysis of the \square form, thus preventing its conversion into the \square form. Furthermore, 6PGL guards against the accumulation of \square -6-phosphogluconolactone, which may be toxic through its reaction with endogenous cellular nucleophiles.

The presence of plant traits in the *Trypanosomatidae*

V. Hannaert, J.-P. Szikora, P. Michels and F. Opperdoes, in collaboration with D. Rigden, CENARGEN/EMPRAPA, Brasilia, Brazil

While searching for fructose-1,6-bisphosphatase in trypanosomatid genome databases, we have recently identified in *T. brucei* a complete open-reading frame encoding a homologue of a related enzyme, sedoheptulose-1,7-bisphosphatase (SBPase). The gene has been cloned and sequenced and

contains a PTS1, making it a glycosomal protein. SBPase is an enzyme typical of the Calvin cycle of photosynthetic organisms and hence (so far) only encountered in the chloroplasts of green algae and plants. Phylogenetic analysis shows that the closest affiliation of the trypanosome enzyme is with that of the chlorophyte *Chlamydomonas reinhardtii*.

Our recent observation that the glycosomal fructose-bisphosphate aldolase is also closely related to its homologues from plants, which all have a broad substrate specificity and are able to synthesize (and cleave) sedoheptulose 1,7-bisphosphate, suggests to us that these two enzymes must function in tandem in the trypanosomatid HMP. A closer inspection of other genes available in the trypanosome genome database revealed many more sequences with either plant or chloroplast/cyanobacterial affiliation. Most of these enzymes fulfil usually functions in either the Calvin cycle, in glycolysis or in the HMP. We hypothesize that these genes of carbohydrate metabolism probably entered the trypanosomatid ancestor from an algal endosymbiont and that their gene products have later been relocated from the endosymbiont to the host, often to its peroxisomes, after which the remainder of the endosymbiont was lost (9). This may explain the enigmatic presence of glycosomes (i.e. peroxisomes specialized in carbohydrate metabolism) in these organisms.

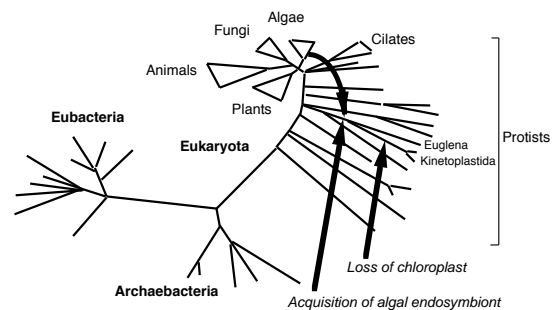


Fig. 2. The "tree of life" based on 16S ribosomal RNA sequences, as modified from Sogin.

Indicated are the supposed acquisition of an algal endosymbiont by an organism ancestral to both euglenoids and kinetoplastids. The subsequent loss of this endosymbiont (and its chloroplast) from the kinetoplastid (*Trypanosoma* and *Leishmania*) lineages took place after their separation from the euglenoids, which still have chloroplasts.

Analysis of the control of the glycolytic flux

M.-A. Albert and P. Michels in collaboration with B. Bakker and H. Westerhoff, Vrije Universiteit Amsterdam, The Netherlands, and S. Helfert and C. Clayton, Universität Heidelberg, Germany

Previously, a mathematical model of trypanosome glycolysis was developed based on the kinetic data available for the enzymes involved. This model was able to predict successfully the experimentally determined fluxes and metabolite concentrations in trypanosomes. Our present experiments focus on the experimental determination of the flux control coefficients of the various steps of glycolysis either by their titration with inhibitors, or by the regulation of the expression of the genes coding for the respective glycolytic enzymes. To this end *T. brucei* cell lines have been created in which the expression of HXK, PFK, TIM and glycerol-3-phosphate oxidase (GPO), the mitochondrial enzyme responsible for oxidising the glycolytically produced NADH, can be decreased by RNA interference (RNAi). For that purpose, double-stranded RNA corresponding to the mRNA is synthesised from a transgene under the control of a promoter that is regulated by exogenously added tetracycline. Using these cell lines, it was shown that the growth rate is halved when the TIM level is decreased to 15%; lower TIM levels are lethal. Indeed, simulation by the model predicted that the flux decreases when the enzyme activity drops to about 30%. Upon lowering the level of GPO mRNA, the oxygen consumption was reduced 4-fold and the rate of trypanosome growth was halved. Similarly, reducing HXK expression by RNAi leads to reduction of the glycolytic flux and the growth rate of the cells. Currently, experiments are in progress to establish the quantitative relationship between HXK and PFK expression on the one hand, and the flux and growth rate on the other hand. Similar experiments are being performed with conditional knockout cells which have been created by disruption of the endogenous HXK and PFK genes after the introduction of a newly introduced additional gene copy under the control of an inducible promoter. These experiments will also reveal if the *in vitro* determined kinetic properties of these enzymes apply also to *in vivo* conditions, and/or if additional regulatory mechanisms occurring in the intact cell have to be invoked.

Biogenesis of glycosomes

J. Moyersoen, H. Krazy, V. Hannaert and P. Michels

Glycosomal matrix proteins are synthesized in the cytosol and imported post-translationally. The translocation of these matrix proteins across the peroxisomal membrane involves a variety of peroxins. Inhibitors interfering with peroxin interactions in trypanosomatids are expected to prevent the synthesis of functional glycosomes and thus kill the parasites. The design of selective inhibitors seems feasible because of the very low level of conservation of peroxins. Previously, we reported the cloning and characterization of two cytosolic peroxins, Pex5 and Pex7. These peroxins act as receptors for proteins to be imported into the glycosomal matrix. Pex5 recognizes the PTS1, a signal specified by the three C-terminal amino acids (10), and Pex7 interacts with the PTS2, a nonapeptide motif close to the N-terminus. Both *T. brucei* peroxins, have been expressed as recombinant proteins in *E. coli*. The functional identity of the 70 kDa *T. brucei* Pex5 has been established *in vitro*; the purified protein recognized glycosomal PGK with high affinity.

We have now also cloned and sequenced the *T. brucei* homologues of Pex6, 10, 12 and 14. Their sequences have 32, 21, 25 and 26% identity with the corresponding human peroxins. Pex14 is part of the receptor-docking complex at the glycosomal membrane. Indeed, we could show, by *in-vitro* experiments, its specific interaction with Pex5. The vital importance of Pex14 for bloodstream-form *T. brucei* was demonstrated by RNAi; induced expression of double-stranded Pex14 mRNA resulted in growth arrest of the cells. Pex10 and Pex12 are possibly involved in the translocation process of the receptors Pex5 and Pex7, charged with their ligand, across the organellar membrane, and/or the dissociation of the receptor-cargo complexes at the matrix site of the membrane. These peroxins contain a C-terminal zinc-binding RING domain known to be involved in protein-protein interactions. Pex6 belongs to the family of AAA-proteins (ATPases Associated with a variety of cellular Activities). It is a large (>100 kDa) protein thought to be either involved in membrane fusion processes which lead to the formation of mature organelles or in the receptor recycling from the organellar matrix to the cytosol. So far, we have expressed in *E. coli* the separate ATPase domain of *T. brucei* Pex6. Crystallization trials are currently being performed with this trypanosome Pex6 domain and with various forms of Pex14 and Pex5, and

with combinations of these latter two peroxins. (J. Choe and Prof. W. Hol).

Analysis of glycosomal membrane solute

Transporters

C. Yernaux and P. Michels

We have started an investigation of glycosomal membrane proteins that might be involved in the transport of glycolytic intermediates or other solutes across the membrane. We have cloned and sequenced two genes coding for putative *T. brucei* glycosomal membrane transporters (TbGAT1 and 2). The amino-acid sequences encoded by these genes are only 30% identical to each other. They are so-called half ABC transporters, containing only a single ATP-binding cassette in their C-terminal half. They are homologous to peroxisomal ABC transporters. Segments of both polypeptides have been expressed in *E. coli* and are being purified; they will be used for antisera production. Investigation of the subcellular localisation and topology of the transporters is in progress. These studies involve transfection of trypanosomes with constructs encoding fusions of segments of the transporters and fluorescent reporter proteins.

Selected publications

1. Michels PA, Hannaert V, Bringaud F. *Metabolic aspects of glycosomes in trypanosomatidae - new data and views*. **Parasitol Today** 2000;16:482-9.
2. Verlinde CL, Hannaert V, Blonski C, Willson M, Perie JJ, Fothergill-Gilmore LA, Opperdoes FR, Gelb MH, Hol WG, Michels PA. *Glycolysis as a target for the design of new anti-trypanosome drugs*. **Drug Resist Updat** 2001;4:50-65.
3. Opperdoes FR, Michels PA. *Enzymes of carbohydrate metabolism as potential drug targets*. **Int J Parasitol** 2001;31(5-6):482-90.
4. Lopez C, Chevalier N, Hannaert V, Rigden DJ, Michels PA, Ramirez JL. *Leishmania donovani phosphofructokinase. Gene characterization, biochemical properties and structure-modeling studies*. **Eur J Biochem** 2002;269:3978-89.
5. Willson M, Sanejouand YH, Perie J, Hannaert V, Opperdoes F. *Sequencing, modeling, and selective inhibition of Trypanosoma brucei hexokinase*. **Chem Biol** 2002;9:839-47.
6. Claustre S, Denier C, Lakhdar-Ghazal F, Lougare A, Lopez C, Chevalier N, Michels PA, Perie J, Willson M. *Exploring the active site of Trypanosoma brucei phosphofructokinase by inhibition studies: specific irreversible inhibition*. **Biochemistry** 2002;41:10183-93.
7. Hannaert V, Yernaux C, Rigden DJ, Fothergill-Gilmore LA, Opperdoes FR, Michels PA. *The putative effector-binding site of Leishmania mexicana pyruvate kinase studied by site-directed mutagenesis*. **FEBS Lett** 2002;514:255-9.
8. Miclet E, Stoven V, Michels PA, Opperdoes FR, Lallemand JY, Duffieux F. *NMR spectroscopic analysis of the first two steps of the pentose-phosphate pathway elucidates the role of 6-phosphogluconolactonase*. **J Biol Chem** 2001;276:34840-6.
9. Hannaert V, Saavedra E, Duffieux F, Szikora JP, Rigden DJ, Michels PA, Opperdoes FR. *Plant-like traits associated with metabolism of Trypanosoma parasites*. **Proc Natl Acad Sci USA** 2003;100:1067-71.
10. Kumar A, Roach C, Hirsh IS, Turley S, deWalque S, Michels PA, Hol WG. *An unexpected extended conformation for the third TPR motif of the peroxin PEX5 from Trypanosoma brucei*. **J Mol Biol** 2001;307:271-82.