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The Evolution of Kinetoplastid Glycosomes

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The available data on carbohydrate metabolism in Kinetoplastida have been reviewed. Based on the metabolic pattern of different kinetoplastid organisms, on the subcellular distribution of their glycolytic enzymes, and on the structural and regulatory properties of these proteins, we propose that the glycosome developed from an endosymbiont, as a specific manner to control carbohydrate and energy metabolism. It is discussed how the enzymes were subcellularly recompartmentalized during evolution as adaptation to the environment encountered by the organisms.

KEY WORDS: Glycosome; microbody; glycolysis; evolution.

EARLY EVOLUTION OF GLYCOSOMES AND OTHER MICROBODIES

The most prominent feature of glycosomes, microbody-like organelles, is that they contain the majority of the enzymes of the glycolytic pathway [reviewed in the accompanying paper (Hannaert and Michels, 1994) and elsewhere (Opperdoes, 1987; Michels, 1989)]. These organelles have been found in all Kinetoplastida examined, but not in organisms belonging to any other phylogenetic lineage.

Microbodies (peroxisomes, glyoxysomes, and glycosomes), as well as some other organelles such as mitochondria and chloroplasts, are absent from the most primitive eukaryotes (Diplomonads, Microsporidia, Trichomonads). These organisms, all protists living under anaerobic conditions, have been grouped in the superkingdom Archezoa (Cavalier-Smith, 1987). In all other eukaryotic taxa microbodies can be found, although in some lineages the organelles may have been lost secondarily from particular organisms.

The distribution of microbodies over the various major taxonomic groups suggests that these organelles originated in evolution after the divergence of the various Archezoa from the main branch of the eukaryotic phylogenetic tree, but before the development of the lineages of the Kinetoplastida and the Euglenoida. These two latter lineages branched off at approximately the same time (Sogin *et al.*, 1986).

Despite the diversity in enzyme content in the various members of the microbody family, we consider a monophyletic origin for these organelles most plausible. This is based on a few common enzymes, the similar morphology and route of biogenesis (Borst, 1989; Michels and Opperdoes, 1991) and, most importantly, on the conservation of a topogenic signal: all microbodies contain proteins which have a C-terminal tripeptide comprising the amino acids -SKL, or permutations of this sequence with residues having similar physicochemical characteristics (De Hoop and AB, 1992). Although this signal is very simple, and may easily turn up in protein evolution, a multiple, independent origin of such a signal, and particularly of the organellar components required to recognize it, would be unlikely. We propose, therefore, that glycosomes and other microbodies have a common origin.

Several scenarios can be considered for the origin of these organelles. Microbodies may have originated by budding of membrane systems present within an ancient unicellular eukaryotic organism, and engulfing part of the cell content. The newly formed vesicle would subsequently have been evolved into a

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functional organelle. Several theoretical objections can be raised against such a hypothesis. First, how would the newly formed vesicle have acquired the possibility to multiply itself? Stable vertical transmission would have been unlikely. Second, microbodies usually contain large parts of metabolic pathways, or even entire pathways (Borst, 1989). It is difficult to imagine that a functional, complete chain of enzymes, with access to substrates and the possibility to dispose of products, would be present at once in a newly formed vesicle (Borst and Swinkels, 1989; Michels and Opperdoes, 1991). Successive transfer of individual enzymes of a pathway to the new organelle is equally unlikely. Intermediate stages would not be an advantage, but rather a burden to the cell. We prefer, therefore, the alternative hypothesis that microbodies are derived from an endosymbiont. The pathway found in the present-day organelles has been there from the beginning. Most of the enzyme content must have been lost during the evolution of the organelles, together with the entire genome of the endosymbiont. Some genes may have been transferred to the nucleus of the host cell, as happened during the evolution of mitochondria and chloroplasts. The corollary of this scenario is the selective retainment of specific functions in the various classes of microbodies. Nevertheless, new enzymes and functions will certainly have been acquired by each class during the evolution.

We propose that the glycolytic process has been retained in the organelles of organisms belonging to the lineage of the Kinetoplastida, because it must have rendered a selective advantage in their early evolution (see below).

Our preference for the hypothesis of an endosymbiotic origin of microbodies is entirely based on the similarity between their way of multiplication and biogenesis and those of mitochondria and chloroplasts, and on theoretical arguments. Cavalier-Smith (1990) argued that microbodies evolved from an endosymbiotic Gram-positive bacterium, merely using theoretical considerations. No experimental data are available as yet that provide strong evidence for this or alternative hypotheses.

Glycosomes have been found in all Kinetoplastida examined, whether belonging to the bodonids or the trypanosomatids. However, glycosomes have not been detected in organisms of any other taxonomic group, including *Euglena gracilis*. This latter organism contains microbodies that, depending on the growth conditions, are either more similar to classical peroxisomes or to glyoxysomes (Müller, 1975). Therefore, microbodies with glycolytic activity must have provided a selective advantage that was only exploited early in evolution by the ancestral Kinetoplastida. Can the nature of this advantage be inferred from study of glycolysis in the present-day Kinetoplastida?

Three specific aspects of glycolysis and glycolytic enzymes can be distinguished in representatives of the Kinetoplastida. (1) The organization of the pathway enables some species (*Trypanosoma brucei, Phytomonas* sp.) to sustain a very high glycolytic flux with a relatively low concentration of enzymes. (2) Glycolytic enzymes such as hexokinase (HK)[2] and phosphofructokinase (PFK) of Kinetoplastida lack the regulatory properties usually found in the corresponding enzymes of other organisms (Nwagwu and Opperdoes, 1982; Cronin and Tipton, 1985, 1987). (3) The glycolytic enzymes are not necessarily confined to glycosomes, even though the glycolytic flux occurs only through the organelle. Most of the glycolytic enzymes of bloodstream form *T. brucei* and *Phytomonas* are almost exclusively found in the glycosome, but this is not the case in *Leishmania, Crithidia*, and *Trypanoplasma* (Hart and Opperdoes, 1984; Opperdoes, 1981; Opperdoes *et al.*, 1988). The subcellular distribution of these enzymes presumably reflects the overall pattern of carbohydrate metabolism of the cell. Indirect evidence has been presented by Keegan and Blum (1993) that gluconeogenesis occurs in *Leishmania donovani* promastigotes, but no gluconeogenic activity could ever be detected in bloodstream form *T. brucei* (Opperdoes, 1987).

We consider it unlikely that the potential for a high glycolytic capacity offered by glycosomes has played an important role in the early development of these organelles. The capacity for a high glycolytic flux is rather a recent adaptation of certain species to their present, highly specific way of parasitic life (see below). In contrast, the two other features may have been relevant in early evolution.

Glycolysis is a central metabolic pathway that is present, at least in part, in all organisms. The essential catalytic steps of this process must, therefore, have

[2] Abbreviations: HK: hexokinase; PGI: glucosephosphate isomerase; PFK: phosphofructokinase; TIM: triosephosphate isomerase; GAPDH: glyceraldehydephosphate dehydrogenase; PGK: phosphoglycerate kinase.

been developed very early in evolution in ancestors common to all major phylogenetic lineages. Glycolytic enzymes are, in general, among the most conserved proteins. However, two enzymes of the pathway, HK and PFK, display considerably more variation in their structure (Fothergill-Gilmore and Michels, 1993). This variation seems to be related to their role as regulatory enzymes; much variation is also observed in the mechanisms by which their activity is regulated in different phylogenetic groups. Whereas the foundations of the glycolytic pathway were established very early in evolution, it is obvious that the control mechanisms of the process are of much later date. Novel mechanisms developed in different lineages. The function of these control mechanisms is twofold: to adjust the glycolytic flux to the cellular needs for energy and precursors for anabolic processes, and to control the interplay of different pathways, such as glycolysis, gluconeogenesis, and the pentosephosphate pathway, which share some enzymes.

The evolution of the glycosome could be considered as a novel manner to control carbohydrate metabolism. Glycolysis became confined to the organelle, whereas gluconeogenesis and the pentosephosphate pathway were kept in the cytosol. There was no need for the kinetoplastid HK and PFK to develop regulatory mechanisms to prevent energy loss by futile cycling. Even if some regulatory mechanisms were already present, they could be lost. This scenario is supported by the finding that HK, a specific glycolytic key enzyme, is almost exclusively found in the glycosome. The subcellular distribution of other key enzymes of glycolysis and gluconeogenesis (PFK, fructosebisphosphatase) remains to be determined.

One could imagine that an endosymbiont with a full set of glycolytic enzymes and transport mechanisms in its membrane, could easily evolve into the glycosome, whereas most of the host's glycolytic enzymes became predominantly devoted to gluconeogenesis.

The genes for most glycosomal enzymes involved in glycolysis of *T. brucei*, and some corresponding genes of *L. mexicana*, have been cloned and characterized (Hannaert and Michels, 1994). Features typical of eukaryotic glycolytic enzymes could be distinguished in most of the encoded proteins. Moreover, when the sequences were used to construct phylogenetic trees, each one indicated a divergence of the kinetoplastids from all other eukaryotes, early in evolution (Michels and Opperdoes, 1991; Michels *et al.*, 1992). These trees matched those calculated using sequences of ribosomal RNA (Sogin *et al.*, 1986), cytochrome *c* (McLaughlin and Dayhoff, 1973), and pyruvate kinase (I. Ernest and P. Michels, unpublished data), a glycolytic enzyme that in all Kinetoplastida is only found in the cytosol. These results do not provide support for an endosymbiotic origin of the glycosome, and of microbodies in general, but neither are they contradictory with such an origin. Assuming that the glycosome originated from an endosymbiont which has lost its entire genome, some genes for glycosomal proteins may have been transferred to the host's nucleus, but such genes have not yet been detected. We propose that the genes of the original host's nucleus took over the role to code for the majority of the organellar proteins, while the endosymbiont was losing its genome. These proteins of the host thus acquired a glycosomal targeting signal. Such a process could occur gradually and would be similar to what happened with those mitochondrial and chloroplast proteins which are now also encoded by original host proteins (Gray, 1989). Only a small fraction of the nuclear-encoded proteins of these latter organelles is presumably derived from the ancestral endosymbionts. A major difference between the proposed evolutionary scenario for microbodies, and the generally accepted ideas about the development of mitochondria and chloroplasts, is that these latter organelles still contain a reduced genome coding for some highly hydrophobic proteins of their inner membrane. This situation presumably would prevent nonspecific association of these proteins with the other membrane systems of the cell, if they were synthesized in the cytosol (Von Heijne, 1986).

By pulse-labelling studies it has been shown that the glycolytic flux in bloodstream form *T. brucei* goes exclusively through the glycosome; no parallel route exists in the cytosol (Visser *et al.*, 1981). The first seven enzymes of the pathway, responsible for the conversion of glucose into 3-phosphoglycerate, are present in the glycosome. Yet, different isoforms of two enzymes, glyceraldehydephosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK), occur in the cytosol (Hannaert and Michels, 1994). The role of the cytosolic GAPDH remains to be established; cytosolic PGK is only present in the procyclic form of the parasite, where it takes over the function of the glycosomal enzyme which is then repressed

(Osinga *et al.*, 1985; Misset and Opperdoes, 1987). A low amount of glucosephosphate isomerase (PGI) seems also to be present in the cytosol, but this remains to be ascertained (Marchand *et al.*, 1989). The presence of PGI in the cytosol would be in line with the observed low activity of the pentosephosphate pathway in this compartment (Cronin *et al.*, 1989). The subcellular distribution of glycolytic enzymes in *Leishmania* and *Crithidia* seems to be quite different from that in bloodstream form *T. brucei*: considerable activity of most enzymes was also detected outside the glycosome (Opperdoes, 1981; Hart and Opperdoes, 1984). As mentioned above, this activity may be required for a cytosolically located gluconeogenic pathway in these oganisms. The distribution of two enzymes, PGI and triosephosphate isomerase (TIM), in *L. mexicana* has been studied in detail (Kohl *et al.*, 1994; K. Nyame and P. Michels, unpublished data). The cytosolic and glycosomal activity of these enzymes could not be atttributed to proteins with different physicochemical properties, and for each enzyme only a single locus could be detected in the genome. This suggests that in *Leishmania* a single protein can distribute itself over the two cell compartments as a result of either an inefficient glycosome targeting signal, or a specific sorting mechanism that remains to be identified. In either case, the original host gene seems to have retained its function to code for the cytosolic enzyme, and in addition acquired the coding function for the protein in the organelle.

LATER STAGES IN THE EVOLUTION OF THE GLYCOSOME

Most Kinetoplastida examined so far are parasitic organisms, and have been adapted to the highly specific environments in their hosts. Some representatives are monogenetic parasites, involving a single, usually invertebrate host, but others have developed a digenetic parasitism, alternating between an invertebrate and a vertebrate or plant host. The environment encountered by different Kinetoplastida is quite different, and even varies considerably for organisms of a single species during their life cycle (Mukkada, 1985; Cazzulo, 1992). These variations also include the carbon and energy sources. For instance, the various *Leishmania* species and *T. cruzi*, when living in their mammalian host, reside predominantly intracellularly. This environment has a low carbohydrate content, but it is rich in amino acids and in fatty acids. In contrast, these same parasites, and some others, live extracellularly in the insect vector, mostly in the intestinal tract, with highly variable levels of sugars, amino acids, and lipids. Evolutionary adaptation of the organisms to their environment will have been accompanied by optimization of their metabolism to the availability of substrates and oxygen. This must have affected the structural and kinetic properties of the enzymes. Adaptation has also resulted in specific expression patterns for the enzymes of the glycolytic and gluconeogenic pathways, and of the oxidative metabolic complexes in the mitochondrion. In most Kinetoplastida studied each of these metabolic systems is operational during all stages of the cell cycle, although the overall metabolic activity and the relative contribution of each system varies.

T. brucei has occupied a "niche" with highly constant conditions: the mammalian bloodstream. This environment is not only constant with respect to temperature, pH, and ionic conditions, but has also a constant, high concentration of glucose and a sufficient supply of oxygen. This rich carbohydrate environment made gluconeogenesis redundant. Furthermore, it enabled *T. brucei* to develop the capacity to obtain sufficient energy by merely increasing its rate of glycolysis, while repressing the mitochondrial Krebs cycle and oxidative phosphorylation. To enhance its glycolytic flux, the trypanosome used essentially the same strategy as applied by other cells [e.g., microorganisms growing under fermentative conditions, such as *Escherichia coli, Zymomonas mobilis*, and *Saccharomyces cerevisiae*, and mammalian muscle cells (for review, see Fothergill-Gilmore and Michels, 1993)]: the concentration of glycolytic enzymes was increased. The fact that the glycolytic enzymes of Kinetoplastida are already concentrated in the glycosome, and that they lack the common feedback control mechanisms, may have enabled *T. brucei* to achieve the very high glycolytic rate with relatively small adaptations. The glycolytic enzymes in *T. brucei*, responsible for the very high flux, constitute only 9% of the total cellular protein, whereas in yeast and *Z. mobilis* this value can be as high as 50%. Other adjustments that presumably had to be made for the enhanced flux were the increase in substrate carriers (Ter Kuile, 1993) and the development of a specific carrier in the plasma membrane to dispose of

pyruvate, the single end-product of *T. brucei* glycolysis (Wiemer *et al.*, 1992).

Analysis of the amino acid sequences of the glycosomal enzymes of *T. brucei* suggests that an additional adaptation had to be made to sustain the high glycolytic flux. Glycosomal enzymes of Kinetoplastida have, in general, an excess of positively charged residues, leading to a high pI (Misset *et al.*, 1986; Wierenga *et al.*, 1987). This pI is higher than that of the cytosolic enzymes of these cells and the corresponding glycolytic enzymes of any other organism. The glycosomal enzymes of *T. brucei* are even more charged than those of other Kinetoplastida (Swinkels *et al.*, 1988; Kendall *et al.*, 1990; Hannaert *et al.*, 1992; Kohl *et al.*, 1994). It has been hypothesized that the excess of positively charged residues in these proteins evolved to neutralize the negative charge of the phosphorylated glycolytic intermediates in the glycosome (Michels *et al.*, 1991; Hannaert and Michels, 1994). The net charge of the proteins may have a causal relationship with the glycolytic rate, but this remains to be ascertained.

Another trypanosomatid organism that has adapted itself to a carbohydrate-rich environment is *Phytomonas*, living in the phloem and xylem of plants. Indeed, the energy metabolism and carbohydrate metabolism of *Phytomonas* are highly similar to that of bloodstream form *T. brucei*: cultured cells perform glycolysis at an extremely high rate (Sanchez-Moreno, 1992). The major metabolic end-products are ethanol and acetate. Glycosomes are abundantly present in these cells. Subcellular fractionation has shown that most glycolytic enzymes are only present in the glycosome. However, no detailed information is as yet available about the physicochemical and kinetic properties of these enzymes.

EVOLUTION OF SPECIALIZED GLYCOLYTIC ISOENZYMES IN DIFFERENT CELL COMPARTMENTS

For two glycolytic enzymes, GAPDH and PGK, activity could be detected in both the glycosome and cytosol of all Kinetoplastida, including *T. brucei*. Analysis of *T. brucei*, *L. mexicana*, and *C. luciliae* has shown that these activities should be attributed to different isoenzymes. Originally it was anticipated that characterization of these isoenzymes would provide a clue about the evolutionary origin of the glycosome. The Kinetoplastida could have retained the different isoenzymes since the early transformation of the endosymbiont into the glycosome.

The general dual localization of GAPDH and PGK is indicative of an important function. An explanation for the existence of two PGK isoenzymes has been proposed by Opperdoes (1987). As discussed above, the expression of these isoenzymes is developmentally controlled (Osinga *et al.*, 1985; Misset and Opperdoes, 1987). This expression seems to be linked to that of another glycosomal ATP-synthesizing enzyme, phospho*enol*pyruvate carboxykinase. The subcellular PGK distribution may therefore be important in maintaining the ATP-balance within the organelle. With regard to GAPDH, the function of the cytosolic enzyme is unknown as yet. It is doubtful that it plays a major role in gluconeogenesis or in the metabolism of the glyceraldehyde-3-phosphate produced by the pentosephosphate pathway. The cytosolic GAPDH activity in bloodstream form *T. brucei* is in very large excess over that of any enzyme of the pentosephosphate pathway, and gluconeogenesis is completely absent in these cells. The enzyme could possibly be involved in the maintenance of the cytosolic redox potential. The observation that the cell has a large pool of triosephosphates, not directly involved in glycolysis (Visser *et al.*, 1981), may be relevant in this respect.

The PGK isoenzymes in *T. brucei* and *C. luciliae* are encoded by clustered, highly similar genes which originated through gene duplication, followed by gene conversions partially erasing differences that arose in time by genetic drift (Osinga *et al.*, 1985; Le Blancq *et al.*, 1988). The cytosolic and glycosomal isoenzymes are 95% identical. The differences involve the much higher positive charge (+13 versus −1) of the glycosomal protein and a C-terminal extension containing the topogenic signal. The PGK is thus not informative concerning the organelle's origin.

For GAPDH the situation is quite different. Two tandemly linked genes that encode the glycosomal GAPDH in *T. brucei*, *T. cruzi*, and *L. mexicana* were detected. In each organism the two genes were completely identical. A single gene codes for the cytosolic isoenzyme and was located elsewhere in the genome. The glycosomal and cytosolic GAPDH are quite different: in *T. brucei* and *L. mexicana* they share only 56% and 55% amino acid identity, respectively. The glycosomal enzyme has a number of unique insertions, a small C-terminal extension bearing a targeting motif, and an excess of positively charged residues (Michels *et al.*, 1986; Hannaert *et al.*,

1992). Moreover, it has also some unique kinetic features, particularly with respect to its interaction with the coenzyme NAD^+ (Lambeir *et al.*, 1991). Phylogenetic analysis indicated that both isoenzymes are indicative of a primitive eukaryote, but yet only distantly related to each other (Michels *et al.*, 1991; Hannaert *et al.*, 1992). This suggested that their genes had presumably no common origin within the Kinetoplastida, and that one of them was obtained from another organism by horizontal transfer. Analysis of the bodonid *Trypanoplasma borelli* gave support for this notion; only one GAPDH, distributed over both cell compartments, could be detected in this organism (E. Wiemer, V. Hannaert, and P. Michels, unpublished data). It appeared to be the typical glycosomal enzyme that was present. Interestingly, Martin and coworkers detected a GAPDH, unambiguously homologous to the glycosomal enzyme of trypanosomes, in the cytosol of *E. gracilis* (W. Martin, personal communication). The sequences share several unique insertions relative to other GAPDH sequences. *Euglena*, a phototrophic protist, is related to the Kinetoplastida, but it does not contain any glycolytic activity in its microbodies. No other cytosolic GAPDH isoenzymes were found in *E. gracilis*. The data on *T. borelli* and *E. gracilis* suggest that the present-day glycosomal GAPDH of Trypanosomatidae was originally a cytosolic enzyme in the ancestor of the Kinetoplastida. Subsequently it became partially compartmentalized, a situation that still exists in *T. borelli*, and that is comparable to that of various other enzymes such as PGI and TIM in many other Kinetoplastida (see above). In a later stage of evolution a foreign GAPDH gene was acquired by an ancestral organism of the Trypanosomatidae lineage, via horizontal transfer from a different, rather primitive eukaryote. This enabled these organisms to develop GAPDH isoenzymes specialized to their functioning in each of the compartments. The original kinetoplastid enzyme became devoted to glycolysis and was consequently fully compartmentalized in the glycosome. The newly acquired enzyme evolved to perform other functions, in the cytosol, for which different kinetic properties were required. As discussed above, these functions are not yet fully appreciated. Nevertheless, we infer that the enzyme fulfills an important role in the metabolism of the parasites, because it has the same, very slow evolutionary rate as the glycosomal isoenzyme and the GAPDH in other organisms (Hannaert *et al.*, 1992).

The comparative analysis of glycolysis and glycolytic enzymes in different representatives of the Kinetoplastida has led us to infer a scenario for the evolution of glycosomes. It can be envisaged that such a scenario could be used as a model for understanding the evolutionary compartmentalization of different enzyme systems in microbodies of other organisms.

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