Localisation of a 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase in the Mitochondrial Matrix of *Trypanosoma brucei* Procyclics

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Contrary to *Leishmania* spp. and *Trypanosoma cruzi*, *Trypanosoma brucei* bloodstream forms do not synthesise their own sterols but take these compounds in the form of cholesterol directly from the mammalian host. However, procyclic insect stages synthesise ergosterol rather than cholesterol. Here the sub-cellular localisation of the first committed enzyme of this pathway of isoprenoid synthesis 3-hydroxy-3-methylglutaryl-coenzyme A reductase in *T. brucei* procyclics (0.9 nmol. min^{-1}. mg^{-1} protein) was carried out using both cell-fractionation by isopycnic centrifugation and digitonin-titration experiments. The majority of the NADP^+-linked 3-hydroxy-3-methylglutaryl-coenzyme A reductase is a soluble enzyme present in the mitochondrial matrix with some additional membrane-associated activity in glycosomes and possibly in the endoplasmic reticulum. It is suggested that the active metabolism of threonine and/or leucine as preferred 2-carbon source for the incorporation of acetyl units into lipids and/or sterols in the mitochondrion of *T. brucei* procyclics is the explanation for a high 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in these protozoan organelles.

Introduction

Protozoan parasites of the Trypanosomatidae family such as *Trypanosoma cruzi* and *Leishmania* species are capable of the biosynthesis of sterols for their incorporation into membranes. The major sterol in the plasma membrane of *Leishmania* spp. is ergosterol (Goad *et al.*, 1984), while the mammalian host incorporates cholesterol into its plasma membranes. This difference between host and parasite has stimulated the use of several drugs such as azoles, inhibitors of ergosterol biosynthesis in experimental chemotherapy of *Leishmania* (reviewed by Urbina, 1997). In the case of *Trypanosoma cruzi* early inhibitors of the sterol biosynthetic pathway and especially of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase have been used in the case of experimental Chagas' disease (Urbina, 1997). Contrary to *Leishmania* spp. and *T. cruzi*, *Trypanosoma brucei* bloodstream forms do not synthesise their own sterols but take these in the form of cholesterol directly from the mammalian host and subsequently incorporate them intact into their plasma membrane (Venkatesan and Ormerod, 1976). However, procyclic insect stages synthesise ergosterol rather than cholesterol and these cells have been shown to contain the first committed enzyme of this pathway of isoprenoid synthesis: HMG-CoA reductase (Coppens *et al.*, 1995). Recently a gene coding for HMG-CoA reductase of *T. cruzi* has been cloned and sequenced (Peña Diaz *et al.*, 1997). The corresponding amino-acid sequence predicts a soluble enzyme while the HMG-CoA reductases from all other eukaryotic organisms reported so far are membrane-bound proteins (Peña Diaz *et al.*, 1997). Indeed, overexpression of the *T. cruzi* protein in *E. coli* led to the production of a soluble and active enzyme. Moreover, a soluble HMG-CoA reductase from *T. cruzi* epimastigote forms was kinetically characterized and it was initially shown that the enzyme is associated with the glycosomes of this trypanosomatid (Concepción *et al.*, 1998). However, more recent

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immunogold labelling studies using ultrathin sections of *T. cruzi* epimastigotes and polyclonal/monoclonal antibodies generated against *T. cruzi* recombinant HMG-CoA reductase suggested that the enzyme is predominantly located inside the mitochondrial matrix (González-Pacanowska *et al.,* 1999). In this paper we show that in *T. brucei* procyclics only a small fraction of the NADP-linked HMG-CoA reductase is membrane bound and associated with glycosomes (Opperdoes, 1987), while the soluble enzyme which represents the major activity in the procyclic stage is present in the mitochondrial matrix.

Material and Methods

Cell fractionation experiments using sucrose-gradient centrifugation were carried out on *Trypanosoma brucei* stock 427 procyclic trypomastigotes (Brun and Schönenberger, 1979) exactly as described previously (Opperdoes *et al.*, 1981). Digitonin-titration experiments were carried out as described by Heise and Opperdoes (1999). Protein concentrations and marker enzyme activities were determined using described procedures (Opperdoes *et al.*, 1981; Stein *et al.*, 1973; Misset and Opperdoes, 1984; Shapiro *et al.*, 1974; Gbenle *et al.*, 1986). HMG-CoA reductase was determined exactly as described by Shapiro *et al.* (1974). In distribution profiles after isopycnic centrifugation all activities are presented as a normalised frequency with the area under each curve equal to unity (Steiger *et al.*, 1980).

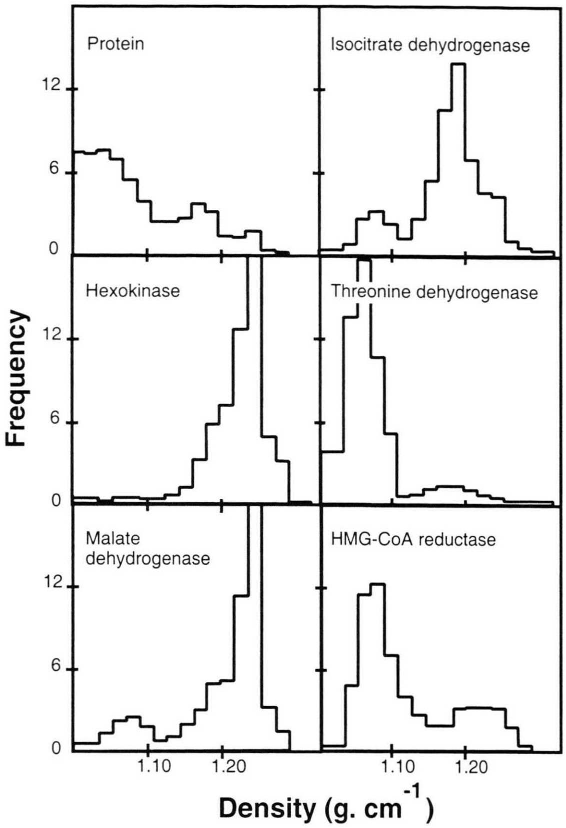

Fig. 1. Equilibrium density distribution of protein content, sub-cellular marker enzymes and HMG-CoA reductase, obtained from cell-free extracts of *T. brucei* procyclics after centrifugation on a linear sucrose gradient. Marker enzymes are hexokinase and malate dehydrogenase for glycosomes and NADP$^+$-isocitrate dehydrogenase and threonine dehydrogenase for mitochondria.

Results and Discussion

The HMG-CoA reductase activity in our cell extracts (0.9 nmol. min^{-1}. mg^{-1} of protein) was intermediate between the values previously reported for *T. brucei* (0.012 nmol. min^{-1}. mg^{-1} of protein) (Coppens *et al.*, 1995) and those reported recently for a soluble extract of *T. cruzi* (3.9 nmol. min^{-1}. mg^{-1} of protein) (Peña Diaz *et al.*, 1997). The sub-cellular localisation of HMG-CoA reductase activity in procyclic forms of *T. brucei*, was carried out using both cell-fractionation by isopycnic centrifugation and digitonin-titration experiments and the results are shown in Figs 1 and 2. The enzyme showed a clear bimodal distribution. Although the majority of the reductase was soluble, the particle-bound activity equilibrated at a density of 1.18–1.24 g cm^{-3} (Fig. 1) suggestive of an association with both mitochondria and glycosomes when compared with the distribution of mitochondrial marker NADP-linked isocitrate dehydrogenase and that of the glycosomal markers hexokinase and malate dehydrogenase. Interestingly the mitochondrial enzyme threonine dehydrogenase (TDH) (Opperdoes *et al.*, 1981) was almost exclusively soluble, with only a small contribution at mitochondrial density (1.18 g cm^{-3}). The bimodal distribution of the HMG-CoA reductase was then verified by a digitonin titration experiment (Fig. 2). Surprisingly, and contrary to the results shown in Fig. 1, digitonin titration did not reveal any sign of neither a cytosolic activity of HMG-CoA reductase nor of TDH. These activities were

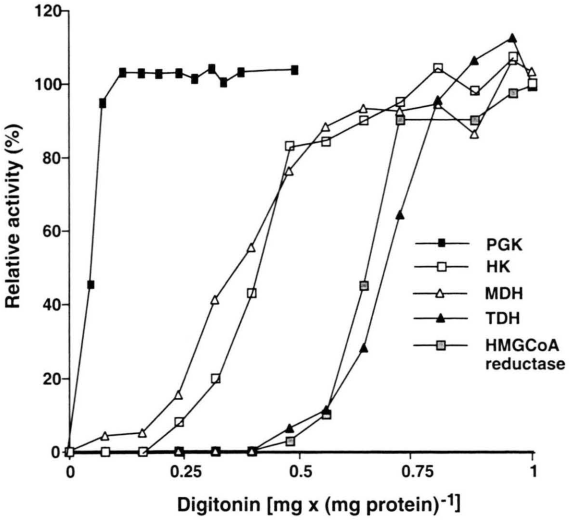

Fig. 2. Subcellular localisation of HMG-CoA reductase activity by digitonin titration. Marker enzymes and maximal specific activities (in nanomoles. $\min^{-1}$. mg^{-1} protein) are phosphoglycerate kinase (PGK, 240) for the cytosol, hexokinase (HK, 50) and malate dehydrogenase (MDH, 200) for glycosomes and threonine dehydrogenase (TDH, 60) for mitochondria.

released only when the mitochondrial inner membrane was permeabilised at the highest concentrations of the detergent used (Fig. 2). In previous experiments using the method of cell grinding for subcellular fractionation we have observed varying contributions of soluble and particulate activities of TDH and of carnitine acetyltransferase, another easily solubilized enzyme (Linstead *et al.*, 1977) involved in the catabolism of threonine in mitochondria (Opperdoes *et al.*, 1981). We interpret the strikingly different results obtained by the two cell fractionation methods to indicate that actually all the TDH activity is present in the mitochondrion as a soluble matrix enzyme and that a considerable part of it can be released by leakage upon grinding of the cells as the result of the rupture of the single tubular mitochondrial network. The results obtained for HMG-CoA reductase should be interpreted in the same way. The different behaviour of TDH and HMG-CoA reductase with that of ICDH, another non membrane bound and matrix associated protein, when using the method of cell grinding for subcellular fractionation (Fig. 1) may reflect a functional organization of the different enzymatic complexes inside the mitochondrial matrix.

The NADP$^+$-dependent HMG-CoA reductase is involved in the formation of mevalonic acid, isoprenoids and sterols and is in mammals associated both with the endoplasmic reticulum and with peroxisomes (Keller *et al.*, 1986; Olender and Simoni, 1982; Engfelt *et al.*, 1997). However, a localisation of HMG-CoA reductase in mitochondria has only been described for rat brain (Patel and Clark, 1981), Leydig cells from rat testis (Pignataro *et al.*, 1983) and recently also for *T. cruzi* (González-Pacanowska *et al.*, 1999). In a previous study, Coppens *et al.* (1995) described in the case of *T. brucei* procyclics an exclusive microsomal distribution of this enzyme, while Peña-Diaz *et al.* (1997) have reported a HMG-CoA reductase from *T. cruzi* that was 95% soluble after sonication and extremely sensitive to proteolytic degradation. Concepcion *et al.* (1998) initially described a HMGCoA reductase that was almost exclusively associated with glycosomes. However, more recent immunogold labelling studies using ultrathin sections of *T. cruzi* epimastigotes and polyclonal/monoclonal antibodies generated against *T. cruzi* recombinant HMG-CoA reductase and digitonin precipitation experiments suggested that the enzyme is predominantly located inside the mitochondrial matrix (González-Pcanowska *et al.*, 1999).

Since our results suggest that there is no cytosolic HMG-CoA reductase in *T. brucei*, all the soluble activity as measured in Fig. 1 must be the result of leakage from the matrix of ruptured mitochondria, as is also the case with the typical mitochondrial enzyme TDH (Opperdoes *et al.*, 1981). The fact that no reductase was released from the cells at low digitonin concentrations does not necessarily mean that compartments other than the mitochondrion do not contain HMG-CoA reductase activity. If such activity would be present in either glycosomes or microsomes it would most likely be an integral membrane protein, as has been reported for the mammalian enzyme, and thus would not be solubilised. It therefore cannot be detected in our digitonin-titration experiment. This interpretation is entirely in agreement with the results of Peña-Diaz *et al.* (1997), who have cloned and sequenced the soluble reductase from *T. cruzi*. The enzyme lacks the typical membrane-spanning N-terminal domain, but a careful inspection of the N terminus of the protein reveals a mitochondrial transit sequence (cf. Häusler *et al.*, 1997). which was as such recognised when the sequence was submitted to the Psort II program available on the Web (http://psort.nibb.ac.jp; Fujiwara *et al.*, 1997). This and our data contrast with the observation that HMG-CoA reductase activity in *T. cruzi* was primarily located in the glycosomal matrix but there was no evidence for a reductase activity in the endoplasmic reticulum (Concepción *et al.*, 1998). Both in *T. cruzi* (Peña-Diaz *et al.*, 1997; Concepción *et al.*, 1998) and in *T. brucei* (this paper) the HMG-CoA reductase activity was several orders of magnitude higher than that reported by Coppens *et al.* (1995) and thus any minor contribution in reductase activity by an ER enzyme could easily have been missed here. Based on the above, we interpret our experiments to indicate that the majority of the HMG-CoA reductase in *T. brucei* is present in the mitochondrial matrix and that in addition there is some membrane-associated activity in glycosomes and possibly in the endoplasmic reticulum.

As to the function of the mitochondrial HMG-CoA-reductase, Ginger *et al.* (1996) have reported that in trypanosomatids leucine rather than acetate itself is the preferred carbon source for the incorporation of acetyl units into sterols. Another possibility is the use of the threonine pathway which converts this aminoacid into equimolar amounts of acetate and glycine in *T. brucei* (Linstead *et al.*, 1977). In the cultured procyclics the acetyl-CoA derived from threonine serves as the preferred source of 2-carbon units for the synthesis of lipids, even in the presence of excess amounts of acetate (Klein and Linstead, 1976). Since the catabolism of both leucine and threonine is mainly a mitochondrial process and since one of the intermediates of this catabolism is HMG-CoA, the active metabolism of leucine and/or threonine in the mitochondrion of *T. brucei* procyclics may be the explanation for a high HMG-CoA reductase activity in these organelles as well.

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