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

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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* procycls (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* procycls 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
immunogold labelling studies using ultrathin sections of *T. cruzi* epimastigotes and polyclonal/mo­
nclonal antibodies generated against *T. cruzi* re­
combinant HMG-CoA reductase suggested that the enzyme is predominantly located inside the mitochondria­nal 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 *Trypa­
osoma brucei* stock 427 procyclic trypomastigotes (Brun and Schönenberger, 1979) exactly as de­
scribed previously (Opperdoes et al., 1981). Digi­
tonin-titration experiments were carried out as de­
scribed by Heise and Opperdoes (1999). Protein concentrations and marker enzyme activities were determined using described procedures (Opp­
erdoes 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 fre­
cuency with the area under each curve equal to unity (Steiger et al., 1980).

**Results and Discussion**

The HMG-CoA reductase activity in our cell ex­
tracts (0.9 nmol min⁻¹ mg⁻¹ of protein) was in­
termediate between the values previously re­
ported 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 isopyc­
nic centrifugation and digitonin-titration experi­
ments 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 glycososomal 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
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; Ölender 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-Pa-canowska et al., 1999). In a previous study, Coppens et al. (1995) described in the case of T. brucei procycls 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 HMG-CoA 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

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.
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 glycososomal 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 amino acid into equimolar amounts of acetate and glycine in T. brucei (Linstead et al., 1977). In the cultured procycls 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 procycls may be the explanation for a high HMG-CoA reductase activity in these organelles as well.

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