

Short communication

Identification of 2-enoyl coenzyme A hydratase and
NADP⁺-dependent 3-hydroxyacyl-CoA dehydrogenase activity
in glycosomes of procyclic *Trypanosoma brucei*

Erik A.C. Wiemer^{1,a}, Lodewijk IJlst^b, Joris van Roy^a, Ronald J.A. Wanders^b,
Fred R. Opperdoes^{a,*}

^aResearch Unit for Tropical Diseases, International Institute of Cellular and Molecular Pathology and Laboratory of Biochemistry,
Catholic University of Louvain, Brussels, Belgium

^bDepartment of Pediatrics and Clinical Chemistry, University Hospital, Amsterdam, The Netherlands

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A common feature of microbodies (i.e. peroxisomes, glyoxysomes) is their capability to catalyse the β -oxidation of fatty acids [1]. In plants and eukaryotic microorganisms, like yeast and fungi, peroxisomes are the only site for fatty acid β -oxidation, while in mammalian cells the peroxisomal β -oxidation pathway has a functional counterpart in the mitochondrion. In organisms where both pathways are present the peroxisomal system seems better suited to degrade very long chain and certain branched chain fatty acids and is also instrumental in the catabolism of

dicarboxylic acids, prostaglandins, leukotrienes and several xenobiotics with acyl side chains [1].

After its activation by the formation of a CoA thioester (see Ref. [2] and references therein), the fatty acid is oxidised in four consecutive enzymic steps. The first reaction is catalysed by a flavoprotein which transfers electrons from an acyl-CoA derivative, via FAD, to O₂. The dehydrogenation of acyl-CoA to *trans*-2-enoyl-CoA is thus coupled to formation of H₂O₂. The latter is converted to H₂O and O₂ by catalase. The second and third reactions are catalysed by a single multifunctional enzyme, possessing the enzymic activities of 2-enoyl-CoA hydratase and NAD⁺:3-hydroxyacyl-CoA dehydrogenase [3]. The L-3-hydroxyacyl-CoA formed in the hydration reaction is subsequently converted into the

* Corresponding author. Tel.: +32 2 7647439; fax: +32 2 7626853; e-mail: opperdoes@trop.ucl.ac.be

¹ Present address: Institute for Hematology, Erasmus University Rotterdam, Rotterdam, The Netherlands.

3-ketoacyl-CoA by the action of the dehydrogenase. The last enzyme is 3-ketoacyl-CoA thiolase which cleaves off acetyl-CoA and consequently shortens the fatty acid by two C-atoms. In mammals recurring peroxisomal β -oxidation cycles give rise to fatty acids with shorter chain lengths that can be oxidized to completion in mitochondria. In plants and lower eukaryotes fatty acids are completely degraded in the peroxisome.

The β -oxidative pathway(s) present in Kinetoplastida, to which the parasitic genera *Trypanosoma* and *Leishmania* belong, are very poorly characterised. Nevertheless, the physiological significance of the catabolism of fatty acids may be considerable in certain life-cycle or developmental stages of the parasites. Non-esterified fatty acids ($C_{12:0}$ – $C_{20:0}$) appear to be an important energy substrate for *Leishmania mexicana mexicana* amastigotes, whereas the utilisation of fatty acids in *L. mexicana* promastigotes occurs mainly for anabolic purposes [4]. Indeed the specific activities of the β -oxidation enzymes 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and thiolase were found to be significantly higher in amastigotes than in promastigotes [5]. Most of the 3-hydroxyacyl-CoA dehydrogenase and thiolase activities were present in mitochondria and only a relatively small percentage was associated with glycosome-enriched fractions [5]. A similar conclusion was reached by Taylor and Gutteridge who investigated the subcellular distribution of some β -oxidation enzymes in *Trypanosoma cruzi* [6]. More conclusive experiments were obtained from isopycnic centrifugation studies with cell-free extracts of *Leishmania major* and *Leishmania donovani*. A 3-hydroxybutyryl-CoA dehydrogenase activity was found to be partly associated with an organellar fraction with a buoyant density of 1.23 g.cm^{-3} , supposedly glycosomes [7]. In these experiments the enzyme palmitoyl-CoA synthetase displayed a bimodal distribution interpreted as possibly mitochondrial and glycosomal in nature [7]. Although the data are fragmentary, one may conclude that these results, when taken together, suggest the existence of both a glycosomal and a mitochondrial β -oxidation pathway in Kinetoplastida, analogous to the situation encountered in mammalian cells.

In contrast to the bloodstream form of *Trypanosoma brucei*, which is totally dependent on glycolysis (see [8] and references therein), the procyclic (insect) life-stage form is capable of deriving its energy from the oxidation of amino acids and fatty acids [9]. A post-nuclear supernatant was prepared from a cell homogenate of procyclic stages of *T. brucei* and fractionated by equilibrium centrifugation on a linear sucrose gradient. The gradient was immediately characterised by measuring protein content (not shown) and marker enzymes for different subcellular compartments. As shown in Fig. 1 the position of the various organelles could be ascertained by determination of the respective marker enzymes: isocitrate dehydrogenase (mitochondria) [10]; hexokinase (glycosomes) [11,12]; α -glucosidase (plasma membrane) [13,14]; α -mannosidase (lysosomes) [15]; acid phosphatase (flagellar pocket) [13]; and the cytosolic marker alanine aminotransferase [15]. The glycosomal marker bands at a density of 1.21 – 1.24 g.cm^3 , clearly distinct from the bulk of protein and the mitochondria.

The subcellular distribution of several (glycosomal) β -oxidation enzymes was determined using assay conditions developed for mammalian cell lysates. We were not able to measure any significant acyl-CoA oxidase activity in any of the subcellular fractions. Since catalase is not present in glycosomes of *T. brucei* [8] to detoxify the H_2O_2 formed by a putative acyl-CoA oxidase, we investigated whether there might in fact be an acyl-CoA dehydrogenase. However, all attempts to detect such an activity were unsuccessful. We subsequently measured enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities in the fractions from the gradient. The activity of enoyl-CoA hydratase in the post-nuclear supernatant using crotonoyl-CoA (C4) was 14-fold higher [$0.5 \text{ Units} \times (\text{mg protein})^{-1}$] than with octenoyl-CoA (C8) as substrate. With both substrates a broad activity profile was observed. With the C4 substrate most of the activity was localized in the mitochondrial fractions (cf. isocitrate dehydrogenase), but with the C8 substrate a considerable amount was associated with the glycosomes (cf. hexokinase).

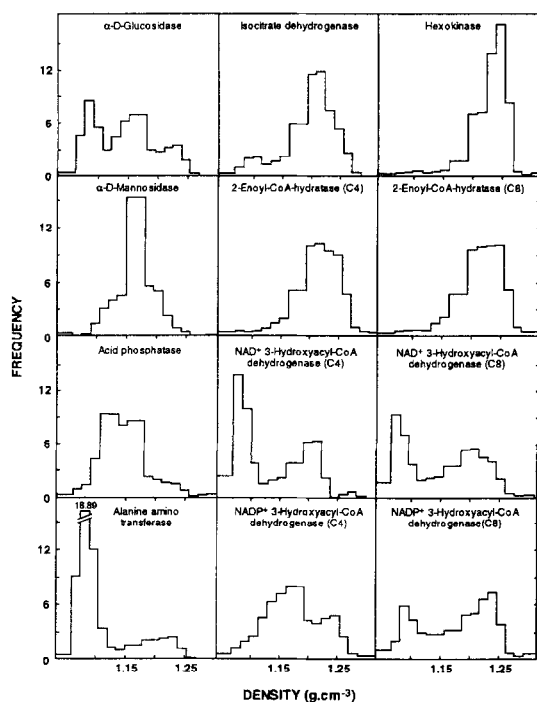


Fig. 1. Distribution profiles of marker enzyme and β -oxidation enzyme activities after isopycnic centrifugation of a post-nuclear supernatant of procyclic trypanosomes. *T. brucei* procyclic forms (stock 427) were cultured in SDM-79 as described by Brun and Schönerberger [23]. Cell homogenates were prepared as described in [13]. Post-nuclear fractions were separated by isopycnic sucrose density gradient centrifugation and analysed as described previously [12]. The fractions were assayed for protein concentration and the following marker enzyme activities: mitochondria (isocitrate dehydrogenase); glycosomes (hexokinase); plasma membranes (α -glucosidase); lysosomes (α -mannosidase); flagellar pocket (acid phosphatase); cytosol (alanine aminotransferase). The β -oxidation enzymes 2-enoyl-CoA hydratase and NAD(P)-dependent 3-hydroxyacyl-CoA dehydrogenase were assayed essentially as described in [24,25], respectively. All β -oxidation enzyme analyses were carried out on a Cobas-centrifugal analyser (Hoffmann La Roche, Basel, Switzerland). The presentation of the distribution profiles is as described by Beaufay and Amar-Costesec [26]. The recovery percentages of enzymes in the gradient were as follows: acid phosphatase: 100; α -mannosidase: 93; α -glucosidase: 103; hexokinase: 99; isocitrate dehydrogenase: 90; alanine aminotransferase: 108; 2-enoyl-CoA hydratase: 100 (C4 substrate) and 98 (C8 substrate); NAD-dependent 3-hydroxyacyl-CoA dehydrogenase: 88 (C4 substrate) and 78 (C8 substrate); NADP-dependent 3-hydroxyacyl-CoA dehydrogenase: 91 (C4 substrate) and 145 (C8 substrate).

In the case of 3-hydroxyacyl-CoA dehydrogenase little activity was measured with NADH as cofactor [about $3 \text{ mU} \times (\text{mg protein})^{-1}$]. This activity was associated with mitochondria (cf. isocitrate dehydrogenase) in addition to a soluble component (cf. alanine aminotransferase), but none of it was associated with glycosomes when either of the two substrates was used. When the measurements were repeated with NADPH as cofactor, significant activities were detected in the post-nuclear supernatant of 30 and $20 \text{ mU} \times (\text{mg protein})^{-1}$ with the C4 and the C8 substrates, respectively. The activity profiles in the gradient exhibited peak activities at densities of 1.24 and $1.16\text{--}1.18$ with the C4 substrate and 1.24 and 1.08 with the C8 substrate, respectively. The presence of an NADP-dependent 3-hydroxyacyl-CoA dehydrogenase activity, active on both substrates in the high-density fractions, strongly suggests a glycosomal localisation of this NADP-dependent activity. Some activity (as measured with the C8 substrate) is also associated with the mitochondrial fractions (1.20) in addition to some soluble activity (1.08). The activity in the low density fractions ($1.16\text{--}1.18$) measured with the C4 substrate is probably associated with membranes of the endoplasmic reticulum.

The bloodstream form of *T. brucei* is dependent on the host for its fatty acid supply. They absorb, interconvert and esterify C16 and C18 saturated and unsaturated fatty acids prior to their incorporation in membranes. However, they are unable to use fatty acids as a source of energy, because they lack a functional citric-acid cycle and cytochrome system. The procyclic insect-stage trypanosomes, on the other hand, have a completely functional mitochondrion, but still fatty-acid degradation occurs at a very low level [16]. Our results show that *T. brucei* procyclics contain a mitochondrial as well as a glycosomal 2-enoyl CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, enzymes that are both involved in β -oxidation of fatty acids. It remains to be elucidated whether the two glycosomal activities are catalysed by a single bifunctional protein, as is the case in the peroxisomes of other organisms, or that these activities result from two different enzymes. Also it is not yet clear whether the soluble dehydrogenase activity represents a true cytosolic enzyme or that the

activity was released from a subcellular compartment upon rupture of the cells. Both the glycosomal hydratase and the dehydrogenase have a preference for long-chain fatty acids. In addition, the glycosomal 3-hydroxyacyl-CoA dehydrogenase is specific for NADP, rather than NAD. The latter finding is surprising since to our knowledge a genuine NADP-dependent 3-hydroxyacyl-CoA dehydrogenase has been identified only in *Clostridium kluyveri* [17,18]. In Clostridia the enzyme catalyses the reduction of acetoacetyl-CoA being the first committed step towards the synthesis of butyrate and butanol. In another *Clostridium* species (*C. beijerinckii*) [19] and in *Mycobacterium smegmatis* [20] a 3-hydroxybutyryl-CoA dehydrogenase was reported which could use either NAD or NADP as cofactor but displayed a higher affinity for NAD.

The finding of NADP-linked activity is intriguing and raises the question as to how the NADPH produced in the glycosome is reoxidized. One possibility is that reoxidation occurs via the ether-phospholipid biosynthetic pathway at the level alkyl/acyldihydroxyacetone-phosphate reductase, an enzyme that has been found in the glycosomes of *T. brucei* procyclics and which accepts NADP as co-factor [21]. Another possibility is that the reoxidation is brought about by the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase, an NADP-dependent enzyme that is associated with both the endoplasmic reticulum and peroxisomes of other organisms and which is active in *T. brucei* procyclics as well [22].

Future experiments are aimed at elucidating the glycosomal β -oxidation pathway which requires development of improved methods of organelle separation plus the isolation of the enzymes involved. Such studies are underway.

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