Involvement of the glycosome of *Trypanosoma brucei* in carbon dioxide fixation

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1. INTRODUCTION

Trypanosomatids are protozoan haemoflagellates which are characterized by at least two unique features: the exceptional organization of their mitochondrial DNA, the kinetoplast DNA [1]; and the presence of microbody-like organelles called glycosomes [2]. These glycosomes contain the early enzymes of the glycolytic pathway, adenylate kinase and orotate phosphoribosyltransferase and orotidine-5′-phosphate decarboxylase, two enzymes of the pyrimidine biosynthetic pathway [3–6]. In *Trypanosoma brucei*, the causative agent of sleeping sickness in man and of nagana in cattle, some of these enzymes are subject to rather drastic changes during the trypanosome's life cyle [6]. For example:

(i) The overall rate of glycolysis, which changes with transformation, is regulated by changes in the glycosomal hexokinase activity;
(ii) Phosphoglycerate kinase activity moves from glycosome towards the cytosol upon transformation from bloodstream form to cultured procyclic trypomastigote;
(iii) A decrease in the activity of the dihydroxyacetone phosphate glycerol-3-phosphate shuttle, functioning in the reoxidation of glycolytically produced NADH, is associated with the appearance of a very active glycosomal malate dehydrogenase.

Here, we report that the enzyme phosphoenolpyruvate carboxykinase (PEPCK), only active in the cultured procyclic form [7], is located inside the glycosome and we propose that together with malate dehydrogenase it constitutes the glycosomal part of a pathway involved in the reoxidation of glycolytically produced NADH and the formation of succinate, one of the end-products of glucose metabolism [8].

Abbreviation: PEPCK, phosphoenolpyruvate carboxykinase

2. MATERIALS AND METHODS

Trypanosoma brucei stock 427 was grown as cultured procyclic trypomastigotes in SDM-79 medium at 28°C [9] and harvested as described. Preparation of cell homogenates, cell fractionation by differential centrifugation and isopycnic sucrose-gradient centrifugation and the presentation of the results were exactly as in [6,10,11].

Hexokinase (EC 2.7.1.1), phosphoglycerate phosphomutase (EC 5.4.2.1), enolase (EC 4.2.1.11), pyruvate kinase (EC 2.7.1.40) and malate dehydrogenase (EC 1.1.1.37) were all measured according to [12]. Malate synthase (EC 4.1.3.2) [13], isocitrate lyase (EC 4.1.3.1) [13], aspartate glyoxylate aminotransferase (EC unclassified) [14], alanine glyoxylate aminotransferase (EC 2.6.1.44) [15], aspartate aminotransferase (EC 2.6.1.1) [12], glutamate dehydrogenase (NAD(P) (EC 1.4.1.2 and (EC 1.4.1.4) [12] and fumarate hydratase (EC 4.2.1.2) [16] were all measured according to described procedures. Phosphoenolpyruvate carboxykinase (ATP) (EC 4.1.1.49) was measured essentially as described [7] in a medium containing 0.1 M imidazole buffer (pH 6.6), 50 mM $KHCO_3$, 2 μCi $NaHCO_3$, 1.25 mM ADP, 1.25 mM PEP, 1 mM $MnSO_4$, 2 mM NADH,

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1 mM glutathione, 3 units malate dehydrogenase and ≤ 100 μg protein. When latency of the enzyme was measured, the incubation mixture was supplemented with 0.25 M sucrose. 'Malic' enzyme (malate dehydrogenase:oxaloacetate decarboxylating-$NADP^+$, EC 1.1.1.40) and pyruvate carboxylase (EC 6.4.1.1) were measured according to [7] except that the assay mixtures were supplemented with 1 mM glutathione. Protein was determined fluorimetrically according to [17] using bovine serum albumin as standard.

All commercially available enzymes, coenzymes and substrates were from Boehringer GmbH (Mannheim). All other chemicals were of the highest purity available.

3. RESULTS

In total homogenates of cultured procyclic trypomastigotes we have measured the specific activities of a number of enzymes involved in glycolysis and intermediary metabolism (table 1). Apart from the early enzymes of the glycolytic sequence, shown in the procyclic trypomastigote [6], phosphoglycerate phosphomutase and enolase were also present but pyruvate kinase could not be demonstrated with a limit of detection of 2 nmol . min^{-1} . mg protein $^{-1}$. Since it has been reported that label from glucose ends up in pyruvate as well as in alanine we have searched for an alternative pathway for the production of pyruvate from glucose.

The enzyme PEPCK was present in large amounts. Its activity was entirely dependent on the presence of PEP as well as on ADP and the latter nucleotide could not be replaced by IDP or GDP. In the absence of Mn^{2+}, magnesium could not restore the activity. Highest activity was found using an imidazole buffer at pH 6.6. 'Malic' enzyme was also present. This enzyme was specific for NADPH; no activity could be detected with NADH. The enzyme was dependent on the presence of Mn^{2+} but this ion could be replaced in part by Mg^{2+} giving 60% of the activity. The enzyme resembled the 'malic' enzyme described for *Crithidia fasciculata* [18] in that it was 22% inhibited by ADP (2 mM), 53% by ATP (2 mM) and 84% by glyoxylate (1 mM). AMP (2 mM) and acetyl coenzyme A (0.25 mM) had no effect. Pyruvate carboxylase activity, if present, was < 2 nmol . min^{-1} . mg protein $^{-1}$ (detection limit).

Table 1

Specific activities of selected enzymes in homogenates of *T. brucei* procyclic trypomastigotes

Enzyme	Specific activity (nmol . min^{-1} . mg protein^{-1})
Phosphoglycerate phosphomutase	40
Enolase	200
Pyruvate kinase	≤ 2
Phosphoenolpyruvate carboxykinase	130
Malate dehydrogenase	1600[a]
Fumarate hydratase	66
Fumarate reductase	72[b]
Malic enzyme (NADP)	35
Pyruvate carboxylase	≤ 2
Aspartate aminotransferase	4
Glutamate dehydrogenase NAD(P)	≤ 1
Aspartate glyoxylate aminotransferase	≤ 2
Alanine glyoxylate aminotransferase	≤ 0.2
Malate synthase	≤ 1
Isocitrate lyase	≤ 1

[a] From [6]; [b] from [7]

Since glyoxylate had a strong inhibitory effect on the malic enzyme from *T. brucei* and since in plants and in *Tetrahymena* sp. the glycoxylate cycle is associated with microbodies, we have tested for the presence of glyoxylate cycle enzymes in *T. brucei*. Apart from malate dehydrogenase none of these enzymes could be detected as reported for the bloodstream form of *T. brucei* [19].

Succinate is one of the end-products of glucose metabolism [8] and the labelling of intermediates is consistent with the operation of the pathway oxaloacetate→malate→fumarate→succinate [7]. The enzymes involved in this pathway: i.e., malate dehydrogenase, fumarate hydratase and fumarate reductase, were all present in *T. brucei* in sufficient activity (table 1) as shown before [7]. Glutamate dehydrogenase, either NAD- or NADP-dependent could not be detected.

The distribution of activities of a number of selected enzymes in fractions obtained after differential centrifugation of homogenates of procyclic culture forms is shown in fig.1. Subsequently a supernatant fraction after 10 min centrifugation at 5000 × *g* was subjected to isopycnic centrifugation on a sucrose gradient (fig.2) as in [6,10]. The results show that enolase and 'malic' enzyme behaved as typical soluble enzymes: they were mainly recovered in the final supernatant (enolase), or remained at the top of the sucrose gradient ('malic' enzyme). PEPCK was entirely sedimentable like the glycosomal marker glycerol-3-P dehydrogenase and both equilibrated at 1.23 g/cm^3 as did the majority of the malate dehydrogenase, another glycosomal enzyme [6]. Aspartate aminotransferase was largely soluble but a significant amount equilibrated at a density typical of mitochondria [6]. No indication was found for an association with glycosomes. In a freshly prepared particulate fraction PEPCK activity was 60% latent whereas malate dehydrogenase exhibited a latency of 96%. Such low latency for PEPCK is probably due to the relatively long incubation time (10 min) during the PEPCK assay, whereas a 2-min incubation in the case of malate dehydrogenase was used.

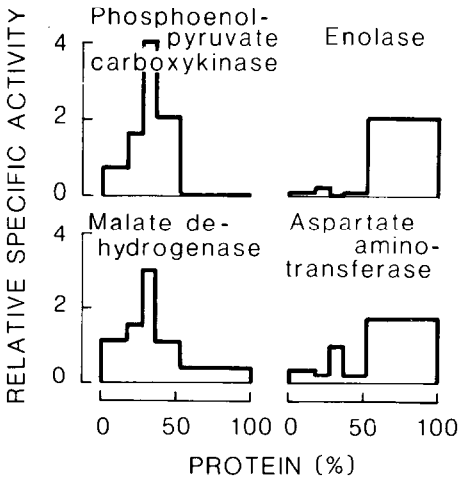

Fig.1. Distribution of enzymes in fractions obtained by differential centrifugation of a homogenate of *T. brucei* procyclics. The recoveries were: phosphoenolpyruvate carboxykinase, 101%; enolase, 142%; malate dehydrogenase, 102%, and aspartate aminotransferase, 109%.

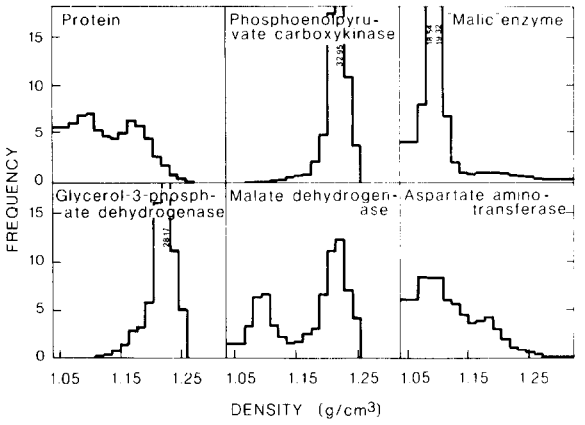

Fig.2. Distribution profiles of enzymes present in a post-large granule extract of *T. brucei* procyclics after isopycnic centrifugation of a linear sucrose gradient. The recoveries were: protein, 114%; phosphoenolpyruvate carboxykinase, 66%; malic enzyme, 72%; glycerol-3-phosphate dehydrogenase (NAD), 105%; malate dehydrogenase, 149%; and aspartate aminotransferase 260%.

4. DISCUSSION

The cultured procyclic forms of the subgenus Trypanozoon catabolise large amounts of glucose.

This glucose consumption is accompanied by the fixation of CO_2 and results in the excretion of significant amounts of succinate under aerobic as well as under anaerobic conditions [8,21]. Members of the Trypanosomatidae contain at least two enzymes capable of the fixation of CO_2; these are PEPCK and 'malic' enzyme [7,22] and based on their respective kinetic and regulatory properties it is assumed that the former enzyme is mainly involved in CO_2 fixation, whereas the latter one is operative in the decarboxylation of malate [7,18,22] resulting in the formation of pyruvate. Our cell-fractionation experiments show that in *T. brucei* both CO_2-fixing enzymes are spatially separated. PEPCK is located inside the glycosome together with malate dehydrogenase and the glycolytic enzymes, whereas 'malic' enzyme is located in the cytosol. Such compartmentation together with the absence of pyruvate kinase from *T. brucei* and the regulatory properties of the CO_2-fixing enzymes would effectively prevent a futile cycling between C_4 dicarboxylic acid and C_3 monocarboxylic acid.

We have suggested that in the cultured procyclic form of *T. brucei* the glycosomal malate dehydrogenase rather than the dihydroxyacetone phosphate glycerol-3-phosphate shuttle [6] is involved in the reoxidation of glycolytically produced NADH. The scheme presented in fig.3 shows how PEPCK and malate dehydrogenase cooperate together in glycosomal ATP synthesis and NADH reoxidation. Contrary to the situation in the bloodstream form essentially all phosphoglycerate kinase in the procyclic trypomastigote is present in the cytosol and not in the glycosome [2,6]. Therefore, the glycosomal ATP consumed in the first part of glycolysis can not be resynthesized by this enzyme and this function apparently has been taken over in the procyclic by PEPCK. To balance glycosomal ATP consumption and production two requirements have to be fulfilled:

(i) All C_3 units leaving the glycosome, probably as 1,3-diphosphoglycerate, have to be forced back

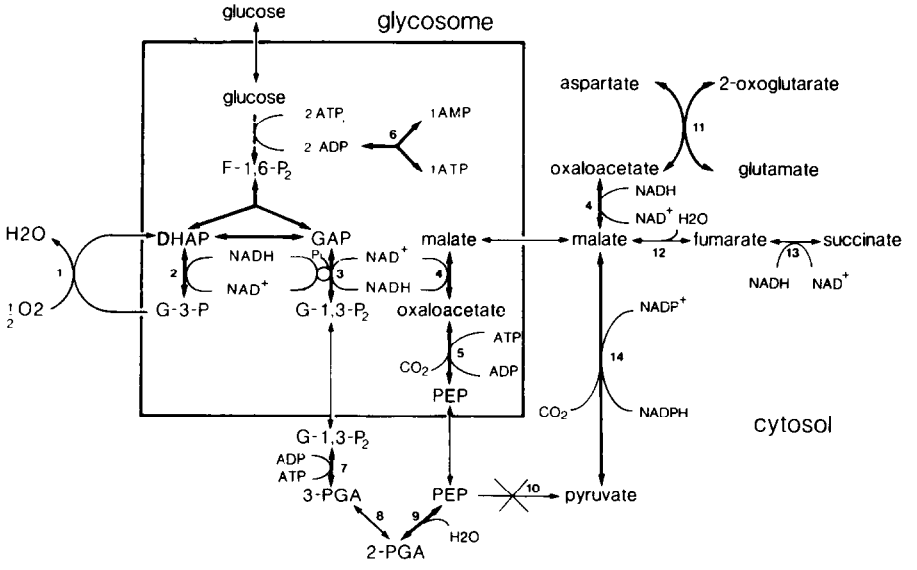

Fig.3. Diagrammatic representation of compartmentation of pathways within *T. brucei* procyclic trypomastigotes. Thick arrows indicate reactions catalysed by enzymes with confirmed subcellular location: (1) glycerol-3-phosphate oxidase; (2) glycerol-3-phosphate dehydrogenase; (3) glyceraldehyde phosphate dehydrogenase; (4) malate dehydrogenase; (5) PEPCK; (6) adenylate kinase; (7) phosphoglycerate kinase; (8) phosphoglycerate mutase; (9) enolase; (10) pyruvate kinase; (11) aspartate aminotransferase; (12) fumarate hydratase; (13) fumarate reductase; (14) 'malic' enzyme.

into the glycosome as PEP to prevent depletion of glycosomal ATP. In such a scheme pyruvate kinase activity should not be detectable, which is exactly what we found.

(ii) All the NADH produced in the glyceraldehyde-3-phosphate dehydrogenase reaction should be available for the reduction of oxaloacetate to malate, otherwise this would result in an accumulation of PEP. This implies that the dihydroxyacetone phosphate glycerol-3-phosphate shuttle, active in the bloodstream form, is inoperative in the procyclic form. At present, no evidence for such a non-functional shuttle is available, since both enzymes involved have been demonstrated in the procyclic form [6].

The scheme in fig.3 indicates that glycosomal CO_2 fixation serves the reoxidation of NADH produced in glycolysis, rather than in some anaplerothic pathway. This agrees with $^{14}CO_2$-labelling experiments [7] which showed an almost complete absence of label from amino acids other than alanine and traces in aspartate, glycine and valine, even after long-term incubations.

Malate is probably an end-product of glycosomal metabolism. We have found no evidence for any fumarase activity associated with glycosomes, nor for any glyoxylate cycle enzyme. Therefore, malate is most likely transported through the membrane into the cytosol where it is converted to other metabolites.

In two distantly related Trypanosomatids, *Trypanosoma cruzi* and *Crithidia fasciculata*, PEPCK was also found to be associated with the glycosome (J.J. Cazzulo and collaborators, personal communication). These observations and ours for *T. brucei* make glycosomal CO_2 fixation most likely to be a general property of all Trypanosomatids.

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