SHORT COMMUNICATION

Intracellular Distribution of Carbon Dioxide-fixing Enzymes in Trypanosoma cruzi and Crithidia fasciculata

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The intracellular distribution of phosphoenolpyruvate carboxykinase (EC 4.1.1.49) and NADP-linked malic enzyme (EC 1.1.1.40) activity in epimastigotes of Trypanosoma cruzi (Tulahuén strain) and in Crithidia fasciculata has been studied by two procedures: (i) subcellular fractionation by differential centrifugation of homogenates obtained by breaking the cells in a mortar; (ii) selective disruption of cellular membranes by digitonin treatment. Phosphoenolpyruvate carboxykinase is particulate in both organisms, as is one of the two forms of malic enzyme present in T. cruzi (malic enzyme I), whereas the other malic enzyme of T. cruzi (malic enzyme II) and the single malic enzyme of C. fasciculata are in the cytosol.

INTRODUCTION

Trypanosoma cruzi, the causative agent of the American trypanosomiasis, Chagas' disease, and the insect trypanosomatid Crithidia fasciculata catabolize glucose only partially to CO2, even under aerobic conditions, with a substantial amount of glucose-carbon being excreted into the medium as organic acids, mostly succinate (Bowman, 1974; Marr, 1973). Synthesis of succinate, which maintains the redox balance of the nicotinamide nucleotide coenzymes (Bowman, 1974), requires fixation of CO2 on a C3 acid (Bowman et al., 1963). Extracts of epimastigotes of T. cruzi contain NADP-linked malic enzyme (EC 1.1.1.40) (Raw, 1959; Cazzulo et al., 1977). We have recently demonstrated two isoenzymes of malic enzyme with different kinetic and regulatory properties (Cannata et al., 1979); malic enzyme II is activated by L-aspartate, whereas the activity of malic enzyme I is not affected by this amino acid (Cannata et al., 1979). Crithidia fasciculata also contains malic enzyme (Marr, 1973), but apparently only one enzyme form, activated by L-aspartate, is present (E. Orellano & J. J. Cazzulo, unpublished results). Both organisms contain ADP-linked phosphoenolpyruvate carboxykinase (EC 4.1.1.49) (Cataldi de Flombaum et al., 1977; Bacchi et al., 1970; Klein et al., 1975), which seems to be the main CO2-fixing enzyme, presumably responsible for the CO2 fixation observed in vivo. In addition, C. fasciculata, but not T. cruzi, contains a low activity of pyruvate carboxylase (EC 6.4.1.1) (Bacchi et al., 1970; Klein et al., 1975; J. J. Cazzulo & B. M. Franke de Cazzulo, unpublished results).

From a physiological point of view, a strict regulation of the activities of phosphoenolpyruvate carboxykinase and malic enzyme is required to avoid a wasteful recycling of the C3-dicarboxylic acid, synthesized by the former enzyme, back to the level of C3-monocarboxylic acid by the decarboxylation reaction catalysed by malic enzyme. Such a regulation could be accomplished through a system of allosteric controls and/or by compartmentation, the two enzymes being located in different subcellular compartments. The results presented

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here suggest that the latter is the case for C. fasciculata, and, at least in part, for T. cruzi; the carboxykinase is strictly particulate and the malic enzyme mostly soluble.

METHODS

Maintenance and growth of the organisms. Trypanosoma cruzi, Tulahuen strain, was grown at 28 °C without shaking in the liquid medium described by Warren (1960), except that bovine serum was used at 4% (v/v) instead of 10%. Crithidia fasciculata, anopheline strain, kindly supplied by Dr S. Hunter of the Haskins Laboratories, Pace University, New York, U.S.A., was maintained and grown as previously described (Higa et al., 1979).

When the cultures of T. cruzi reached a cell density of about 1 × 10⁸ organisms ml⁻¹ (early stationary phase; usually after 6 d), the epimastigotes were harvested by centrifugation (4000 g, 10 min) and then washed once in 0.15 M-NaCl and three times in 20 mM-morpholinopropanesulphonate (MOPS) buffer, pH 7.0/0.25 m-sucrose/3 mM-EDTA solution (MSE) by centrifugation as above. Crithidia fasciculata was harvested and washed in 0.25 m-sucrose/5 mM-KCl solution as previously described (Higa et al., 1979). All harvesting, washing, disruption and fractionation procedures were carried out at 4 °C except where stated otherwise.

Subcellular fractionation. The washed cells of C. fasciculata were broken by grinding with glass powder in a mortar, and the homogenate was centrifuged (14000 g, 15 min) as previously described (Higa et al., 1979). The procedure used for T. cruzi was similar, except for the use of MSE solution and centrifugation at higher speed (30000 g, 15 min). The sediments obtained from both species were washed with half the original volume of the relevant washing solution, and the washings were mixed with their original supernatants (cytosol fraction). The pellets were resuspended in MSE solution (T. cruzi) or in 25 mM-Tris/HCl buffer, pH 7.6, containing 0.5 mM-EDTA (C. fasciculata) and disrupted by sonic disintegration in a Branson sonifier (three treatments, 5 s each at maximum power). The suspensions were then centrifuged (30000 g, 15 min). These supernatants (extracts of the particulate fraction), as well as the original 14000 or 30000 g supernatant (cytosol), were dialysed (6 h, 2 °C) against 20 vol. 50 mM-EDTA buffer, pH 7.6, containing 1 mM-EDTA, and used for the determination of enzyme activities.

Digitonin treatment. Washed cells of both trypanosomatids were suspended in MSE solution (500 to 750 mg wet wt ml⁻¹) and added to MSE solution containing various concentrations of digitonin (Sigma), added as a fresh solution in dimethylformamide (40 mg ml⁻¹); the final volume was 1 ml. After incubation for 5 min at 25 °C (T. cruzi) or for 1 min at 30 °C (C. fasciculata) the suspensions were centrifuged in an Eppendorf microcentrifuge (2 min for T. cruzi or 1 min for C. fasciculata). The supernatants were kept as the cytosol fraction. The pellets were resuspended in 20 mM-sodium phosphate buffer, pH 7.0, containing 3 mM-EDTA and 2 mg digitonin ml⁻¹; after sonic disintegration for 3 s at maximum output of the Branson sonifier, the suspensions were centrifuged again in the Eppendorf microcentrifuge, and the supernatants (extracts of the particulate fraction) were kept.

Assay methods. Protein was estimated by Lowry's method using bovine serum albumin as standard.

Phosphoenolpyruvate carboxykinase was assayed radiochemically by the ¹⁴CO₂-oxaloacetate exchange reaction (T. cruzi) or by direct fixation of ¹⁴CO₂ on phosphoenolpyruvate (C. fasciculata), as previously described (Cataldi de Flombaum et al., 1977) but at pH 5.4. Malic enzyme (Cazzulo et al., 1977) and citrate synthase (Juan et al., 1977) were assayed spectrophotometrically, as previously described.

RESULTS AND DISCUSSION

The particulate fraction of T. cruzi contained 69% of the phosphoenolpyruvate carboxykinase and 23% of the citrate synthase which was used as a marker of the mitochondrial matrix. In the same experiment, 92.4% of the total malic enzyme activity was recovered in the supernatant fraction, with 4.6-fold activation by L-aspartate (determined as described in the legend to Fig. 1), whereas the malic enzyme extracted from the particulate fraction (7.6%) was not activated by L-aspartate. When similar experiments were performed with C. fasciculata, the results were essentially the same. Nearly 100% of the phosphoenolpyruvate carboxykinase activity was recovered in the particulate fraction, which also contained about 38% of the citrate synthase. About 93% of the malic enzyme was recovered in the supernatant fraction, with an L-aspartate activation of 1.9-fold, but the malic enzyme activity remaining in the particulate fraction (7%) was activated by L-aspartate to some extent (1.4-fold). These results, although suggesting a different subcellular localization for the CO₂-fixing enzymes, clearly indicated that considerable fragmentation...
Fig. 1. Activities of phosphoenolpyruvate carboxykinase, malic enzyme and citrate synthase in whole cells of Trypanosoma cruzi (a) and Crithidia fasciculata (b) treated with digitonin. Digitonin treatment and enzyme assays are described in Methods: 250 or 76 mg wet wt ml⁻¹ (corresponding to 15.1 or 3.9 mg protein, respectively) were used in each tube of the Eppendorf microcentrifuge. Open symbols correspond to the supernatant (cytosol) and closed symbols to the extract of the particulate fraction: ○, ●, malic enzyme; □, ■, phosphoenolpyruvate carboxykinase; △, ▲, citrate synthase; ▽, ▼, activation of malic enzyme by 0.5 mM-L-aspartate, determined in a reaction mixture similar to that described by Cazzulo et al. (1977) except for the use of 0.1 mM-L-malate.

of the mitochondria was taking place, as shown by the leakage of citrate synthase into the supernatant. In order to avoid this difficulty we decided to try the procedure developed by Zuurendonk & Tager (1974) for the rapid separation of the cytosol from the particulate components of the cell by treatment with digitonin, which specifically interacts with 3-hydroxysteroids in the plasma membrane. When this method was applied to whole cells of T. cruzi (Fig. 1a) and C. fasciculata (Fig. 1b) the results were in good agreement with those of the subcellular fractionation, but were more clear-cut. Phosphoenolpyruvate carboxykinase and citrate synthase now behaved similarly, and required a considerably higher digitonin concentration for extraction as compared with the bulk of the malic enzyme activity. This procedure also allowed a net differentiation between malic enzymes I and II from T. cruzi (Fig. 1a). The cytosol malic enzyme approached a plateau at 1.5 to 2.0 mg digitonin ml⁻¹, and it was clear that the easily extracted enzyme was the form activated by L-aspartate, malic enzyme II. At 2 mg digitonin ml⁻¹, malic enzyme I was already significantly extracted from particles, as shown by a decrease in the activation by L-aspartate from about 10-fold to about 6-fold. On the other hand, in C. fasciculata (Fig. 1b) practically all the malic enzyme activity was extracted by 2 mg digitonin ml⁻¹, with an activation by L-aspartate of about 2-fold, similar to that observed on extraction of whole cells with digitonin. The low activity of malic enzyme present in the extracts of the 1.5 to 2 mg digitonin ml⁻¹ pellets dialysed against 50 mM-Tris/HCl buffer pH 7.6 containing 1 mM-EDTA was activated by L-aspartate by about 3-fold. These results suggest that C. fasciculata unlike T. cruzi contains only one form of malic enzyme. Polyacrylamide gel electrophoresis followed by staining for enzyme activity under the same conditions as those used to obtain a clear separation of the two isoenzymes of T. cruzi (Cannata et al., 1979) failed to detect more than one enzyme form in C. fasciculata preparations (E. Orellano & J. J. Cazzulo, unpublished results).

Our results show that phosphoenolpyruvate carboxykinase is particle-bound in trypanosomatids, as previously suggested by Klein et al. (1975), but do not allow us to determine whether this enzyme, and also malic enzyme I from T. cruzi, are present in the mitochondrion or in some other organelle, such as the glycosome (Opperdoes & Borst, 1977), which seems to be also present in T. cruzi (Gutteridge & Taylor, 1979). Further studies on this are in progress.

The clear separation into different subcellular compartments of phosphoenolpyruvate
carboxykinase and malic enzyme (in *C. fasciculata*) or malic enzyme II (in *T. cruzi*) should facilitate considerably the regulation of the partial aerobic fermentation of glucose. The L-aspartate-activated malic enzyme, which is more likely to be involved in the degradation of C₄-dicarboxylic acids ([Cannata et al., 1979](#)), is completely separated from the C₄-dicarboxylic acid-synthesizing phosphoenolpyruvate carboxykinase. In the case of *T. cruzi*, part of the problem would remain if malic enzyme I and the carboxykinase were present inside the same particle; however, it must be noted that malic enzyme I is very strongly inhibited by oxaloacetate (*K* of 9 μM; [Cannata et al., 1979](#)) and probably would show little activity under the conditions for the partial aerobic fermentation of glucose, when some increase of the oxaloacetate concentration in the carboxykinase compartment might be expected to occur.

The results presented here suggest that digitonin treatment, previously employed prior to conventional subcellular fractionation studies with *C. fasciculata* ([Kusel & Storey, 1972](#); Edwards & Lloyd, 1977), may become a powerful tool for the determination of subcellular localizations in organisms like the Trypanosomatidae, where the presence of the kinetoplast–mitochondrion complex makes clean subcellular fractionation very difficult.

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