ENZYMES OF PYRIMIDINE BIOSYNTHESIS IN *TRYPANOSOMA CRUZI*

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1. Introduction

We have shown that washed cell suspensions of the three main morphological forms of *Trypanosoma cruzi,* the causative agent for Chagas' disease, incorporated radiolabelled bicarbonate into nucleic acid pyrimidines, suggesting that all forms can biosynthesise these compounds de novo [1]. This conclusion is in agreement with what is known about pyrimidine biosythesis generally in parasitic protozoa [2,3], but differs from that drawn for *T. cruzi* in [4,5]. Here we confirm our conclusion by demonstration that all six enzymes of the pyrimidine biosythetic pathway are present in the three main forms of *T. cruzi.* We also show that the last three enzymes of the pathway have an unusual intracellular distribution.

2. Materials and methods

The preparation of culture epimastigote, blood trypomastigote and intracellular amastigote forms of the Sonya strain of *T. cruzi* has been described [6,7]. To prepare enzyme extracts, organisms (10^9 cells/ml assay buffer) were usually broken by 3 cycles of freezing and thawing. However, for ammonium-dependent and glutamine-dependent carbamoyl-phosphate synthetases (CPSase I and CPSase II, respectively) parasites suspended in water were disrupted in a tight-fitting glass homogeniser (water lysis). All extracts were dialysed before use. Homogenates for cell fractionation were prepared either by grinding with silicon carbide, or by water lysis. The extracts were centrifuged at $2500 \times g_{max}$ for 15 min to remove whole cells and other large debris, then centrifuged at $200\,000 \times g_{max}$ for 20 min to yield particulate and supernatant fractions. All enzyme activities were measured in the physiological direction. Assays for CPSase I and II [8], aspartate carbamoyltransferase (ACTase) [9] and dihydroorotate hydroxylase (ACTase) [9] and dihydroorotate hydroxylase (DHOHase) [10] were based on published methods. The assay of dihydro-orotase (DHOase) measured the production of radioactive dihydroorotate from [^{14}C]carbamoyl-L-aspartate. Orotate phosphoribosyltransferase (OPRTase) was assayed by measuring the production of radioactive OMP and UMP from [5-^{3}H]orotic acid. Orotidine-5′-phosphate decarboxylase (ODCase) activity was assayed by estimating the production of radioactive UMP, uracil and uridine (products of UMP metabolism) from radioactive OMP. Separation of radioactive metabolites was carried out on PE1-cellulose thin layer chromatograms by the method in [11]. Chromatograms were cut up and estimated for radioactivity. Full details of all these procedures will be given subsequently. Protein was assayed by the Lowry method [12].

Abbreviations: ACTase, aspartate carbamoyltransferase (EC 2.1.3.2); CPSase I, carbamoyl-phosphate synthetase (ammonia) (EC 6.3.4.16); CPSase II, carbamoyl-phosphate synthetase (L-glutamine) (EC 6.3.5.5); DHOase, dihydroorotase (EC 3.5.2.3); DHOHase, dihydroorotate hydroxylase (EC unclassified); ODCase, orotidine-5′-phosphate decarboxylase (EC 4.1.1.23) and OPRTase, orotate phosphoribosyltransferase (EC 2.4.2.10)

3. Results

Table 1 shows the specific activities in broken cell preparations of epimastigote, amastigote, and trypomastigote forms of *T. cruzi* for the six enzymes required for pyrimidine biosynthesis de novo. These activities refer to unfractionated homogenates and were all measured at optimal pH in the physiological

Table 1
Specific activities of the enzymes of the pyrimidine biosynthetic pathway in *T. cruzi*

Enzyme	pH optimum	Morphological form		
		Epimastigote (28°C)	Amastigote (37°C)	Trypomastigote (37°C)
CPSase I	8.8	1.5 ± 0.8 (4)[a]	5.2 ± 1.9 (3)	2.9 ± 0.4 (3)
CPSase II	7.1	1.0 ± 0.4 (4)	2.6 ± 1.4 (3)	1.6 ± 0.7 (3)
ACTase	8.5	15 ± 2 (3)	22 ± 3 (2)	31 + 1 (2)
DHOase	6.5	0.09 ± 0.01 (4)	0.16 ± 0.05 (3)	0.25 ± 0.03 (3)
DHOHase	8–9.5	0.38 ± 0.06 (2)	0.26 ± 0.03 (2)	0.37 ± 0.04 (2)
OPRTase	7.4	0.14 ± 0.02 (2)	0.07 ± 0.01 (3)	0.24 ± 0.09 (2)
ODCase	8.5	0.50 ± 0.14 (2)	1.6 ± 0.2 (2)	1.9 ± 0.4 (3)

[a] nmol . min^{-1} . mg $protein^{-1}$

The figures represent means ± SD no. determinations from separate experiments in brackets

direction under conditions of saturating substrate concentrations (based on the reported K_m values for the isofunctional enzymes in other cells, reviewed in [13]). The pH optima were obtained using partially purified enzyme extracts of epimastigotes. The enzymes with the lowest specific activities have optima near to physiological pH. The activity at pH 7.0 of ACTase is 7.5 that of DHOHase, 0.2, that of ODCase, 0.25 and that of CPSase I, 0.07 nmol . min^{-1} . mg $protein^{-1}$. The specific activities of the enzymes within the pathway show great variation (e.g., 0.1-15 nmol . min^{-1} . mg $protein^{-1}$ in epimastigotes). However, each enzyme shows comparatively little difference in activity in the three forms of this organism.

Table 2
Distribution of pyrimidine biosynthetic activities in homogenates of epimastigotes of *T. cruzi*

Enzyme	Total act.[a]	Relative spec. act.[b]	
		Pellet	Supern.
CPSase I	0.05	0.93	1.1
	(2.6)	(0.28)	(2.1)
CPSase II	n.d.	–	–
	(4.2)	(0.24)	(2.2)
ACTase	17	0.12	1.7
	(130)	(0.34)	(2.1)
DHOase	0.35	0.09	2.2
DHOHase	0.10	<0.15[c]	>2.1
OPRTase	0.12	>1.6	<0.23[c]
	(1.2)	>1.6	<0.12[c]
ODCase	0.26	1.6	0.18

[a] nmol . min^{-1} . mg $protein^{-1}$ at 28°C
[b] % total activity/% total protein
[c] Activity not detected; the figure given represents the limit of reproducible detection
n.d., Not detected

The figures in brackets refer to fractions prepared by water lysis; all other results refer to fractions obtained through grinding with silicon carbide

Both CPSase I and CPSase II activities were detected in all forms of *T. cruzi*. *N*-Acetyl-L-glutamate was routinely added to ammonium-dependent assays. However, its requirement could not be demonstrated, possibly because the duration of dialysis was necessarily short (10 min) due to enzyme instability. This may not have removed sufficient endogenous *N*-acetyl-L-glutamate to demonstrate its dependence. The effect of pH on CPSase I and CPSase II activities suggests that, under physiological conditions, glutamine is the more likely ammonium donor for pyrimidine biosynthesis.

The distribution of the biosynthetic enzymes in pellet and supernatant fractions of homogenates of epimastigotes are given in table 2. The results for the preparation obtained by silicon carbide grinding show little detectable CPSase activity. The other enzyme activities are comparable to those obtained by freezing and thawing (table 1). ACTase, DHOase and DHOHase were found to be predominantly in the supernatant fraction, while OPRTase and ODCase, the terminal two enzymes in the pathway, were in the particulate fraction. This distribution has been found routinely by breaking the cells through freezing and thawing.

Using water lysates, high CPSase I and CPSase II activities were found. Both were predominantly in the supernatant fraction, as was ACTase, while

OPRTase was particulate. The increase in specific activities of these three enzymes by this method over those found in table 1 was probably due to more cell protein being pelleted during centrifugation at $2500 \times g_{max}$ for 15 min prior to fractionation. The result for the CPSase I activity from silicon carbide grinding indicates that the enzyme is polydisperse. However, the total activity in this preparation represent such a small % of what is present that it suggests the result is artifactual.

4. Discussion

All the enzyme activities necessary for the *de novo* biosynthesis of UMP were detected in broken cell preparations of amastigote, trypomastigote, and epimastigote forms of *T. cruzi* (table 1). All were measured in the physiological direction and were sufficient to account for the majority of the pyrimidine requirements of the organism (~0.1 nmol . min^{-1} . mg $protein^{-1}$). The relatively low activity for OPRTase from amastigotes (0.07 nmol. min^{-1} .mg $protein^{-1}$) may be due to sub-optimal conditions of preparation. In particular the method of cell breakage can have major effects upon enzyme activity [14] and significant inhibition may be occurring due to the presence of potential inhibitors in the commercial 5′-phosphoribosyl 1-pyrophosphate used in the assay.

The results in table 1 provide a feasible explanation for the incorporation of radioactive bicarbonate into RNA and DNA [1]. Despite the existence of potential salvage pathways [1,4,5], it seems likely that *in vivo* blood and intracellular forms of *T. cruzi* will depend primarily on this *de novo* pathway as a source of pyrimidines. This situation is similar to that described for *Trypanosoma equiperdum* [15].

Both CPSase I and CPSase II activities have been found in the three forms of *T. cruzi*, but at physiological pH, glutamine is likely to be the ammonium donor. Both CPSase I and CPSase II activities are unstable. This observation may explain previous difficulties in assaying these enzymes in *Crithidia fasciculata* [16]. Both enzyme activities are soluble, so that it is possible that there is one enzyme exhibiting both activities, as is found in extracts from other sources [13].

The specific activities of the enzymes of the pyrimidine biosynthetic pathway vary greatly within each form, but there is comparatively little difference between each enzyme in the three forms of *T. cruzi*. Our results thus add more evidence to the suggestion that there is little biochemical difference in the pyrimidine biosynthetic pathways of epimastigote, amastigote, and trypomastigote forms of *T. cruzi* [1].

The high specific activities of both CPSase I and CPSase II (table 1) and ACTase suggests that, unlike the pathways of many other cells, neither CPSase I and CPSase II nor ACTase are likely to be important regulatory enzymes in *T. cruzi*.

In common with other known pyrimidine biosynthetic pathways, CPSase II, ACTase, and DHOase are soluble enzymes. In contrast to other cells, OPRTase and ODCase are particulate enzymes, not only in *T. cruzi* (table 2) but also in *Leishmania mexicana mexicana* [17] and probably in *T. equiperdum* [15]. It is possible that this difference may be a characteristic of the Kinetoplastida, and may allow parasite-specific inhibitors to be found of the type described [15] for the pyrimidine biosynthetic pathway of the members of this order. Such inhibitors could be synergistic with inhibitors of DHOHase. In the Kinetoplastida this enzyme is soluble (table 2 and [10]) and requires oxygen for activity, but is not sensitive to inhibition by CN^- (10 mM) or antimycin A (100 μg/ml). In contrast, in mammalian cells the isofunctional enzyme is a dehydrogenase which is particulate, mitochondrial and intimately connected to the respiratory chain to which it passes electrons directly, probably at the ubiquinone level [10].

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