CARNITINE STIMULATES ATP SYNTHESIS IN *TRYPANOSOMA BRUCEI BRUCEI*

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1. Introduction

The parasitic protozoon *Trypanosoma brucei brucei* is one of the causative agents of African Trypanosomiasis which, as sleeping sickness in man and ngana in cattle, has considerable economic and epidemiological importance [1]. Infections of laboratory rodents with *T. b. brucei* form an excellent source for studies of the biochemistry of this organism and a number of important metabolic differences between the parasite and its host have been identified. Lacking a functional tricarboxylic acid cycle [2], bloodstream forms of *T. b. brucei* are largely dependent on glycolysis for ATP production [3] and the consumption of oxygen is linked to the cyanide-insensitive terminal electron acceptor α-glycerophosphate oxidase [4,5]. Anaerobic metabolism may proceed with a reduced ATP yield, but still depends on glycolysis [3]. It is also known that *T. b. brucei* is able to metabolize threonine to acetyl CoA which can act as a preferred source of carbon for lipid synthesis [6]. Subsequent investigation of possible pathways of acetyl CoA hydrolysis [7] have revealed both high levels of carnitine acetyl transferase (CAT), as well as intracellular carnitine levels of 1–5 mM which are comparable with the highest values reported in biological systems [8,9] and considerably higher than that in rat plasma [10]. The physiological role of carnitine in fatty acid oxidation in mammalian systems is well known, but this oxidative pathway has been reported as absent in trypanosomes [11]. This work describes investigations of a possible role for carnitine and carnitine acetyl transferase in trypanosomes.

Abbreviations: CAT, carnitine acetyl transferase; BAC, bromo-acetyl-carnitine; DTNB, 5,5′-dithio-*bis*-(2-nitrobenzoic acid); DTBP, 4,4′-dithio-*bis*-pyridine

We considered it possible that the CAT stage of the pathway for acetate metabolism could be involved in a substrate-level production of ATP, perhaps making use of the high group-transfer potential of acetyl carnitine [7]. The effects on ATP levels in *T. b. brucei* of two potential trypanocides, MPF15,246 and its biologically active metabolite MPF8,167, and of bromo-acetyl-carnitine (BAC) which is an irreversible inhibitor of CAT from pigeon breast muscle [12], have been used to examine this possible role of carnitine and CAT in these organisms.

2. Materials and methods

2.1. *Trypanosomes*

Rats (CFY strain) were used as laboratory hosts for *Trypanosoma brucei brucei* strain 427. Blood was obtained by aortic puncture and the parasites were separated and harvested as in [13] as adapted from [14].

2.2. *Measurement of steady state ATP levels*

After separation trypanosomes were washed thoroughly and resuspended in incubation buffer (4.99 mM KCl, 80.1 mM NaCl, 2.0 mM $MgCl_2$, 16.2 mM Na_2HPO_4, 3.8 mM NaH_2PO_4 and 1.5 gm/l bovine serum albumin) at a final concentration of 10^7 organisms/ml with various concentrations of L-threonine, D-glucose and L-carnitine as specified in table 1. For ATP measurements the firefly luciferase bioluminescence assay was used [15]; reagents were obtained from Packard and 10 μl trypanosome suspension was added to 50 μl Pico-Ex5 to lyse the cells and release ATP. Samples (1–5 μl) of this extract were then added to 40 μl reconstituted firefly luciferase enzyme in a 6 × 50 mm borosilicate glass tube

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(Kimble, USA) and luminescence measured using a Packard Tricarb 460C liquid scintillation spectrometer adapted for the purpose. The ATP concentration was determined by use of appropriate calibrated ATP standards.

2.3. *Measurement of ATP synthesis*

ATP synthesis was measured in cell extracts prepared by high speed homogenization of a suspension containing $1-5 \times 10^9$ organisms/ml. The medium contained incubation buffer with added ADP (1 mM), ouabain and oligomycin (each 1 mM) to inhibit ATPase activity, and trypanosome homogenate at a final concentration equivalent to 5×10^8 organisms/ml in a total volume of 1 ml. After 5 min at 37°C, 5 μl 10 mM $Na_2H^{32}PO_4$ (initial spec. act. 2 mCi/ml; Amersham International) were added and the incubation continued for a further 15–30 min before the addition of 0.5 ml 35% (v/v) $HClO_4$. After centrifugation 0.5 ml supernatant solution was removed and added to 1 ml 5% (w/v) ammonium molybdate and the mixture left at room temperature for 10 min. To this aqueous solution 2 ml *n*-butyl acetate were added, the 2 phases thoroughly mixed, centrifuged and then 0.1 ml from each layer added to 10 ml scintillation fluid (scintillator 299 Packard Ltd) for the determination of radioactivity. This method is based upon an adaptation [16] of the method in [17] in which P_i is extracted into the butyl acetate phase leaving bound phosphate in the aqueous fraction, rather than using activated charcoal.

2.4. *Measurement of CAT activity*

CAT (acetyl CoA-L-(−)-carnitine-*O*-acetyltransferase, EC 2.3.1.7) activity was measured in cell extracts prepared by trypanosome lysis with 0.5% (w/v) Triton X-100 followed by centrifugation at $40\,000 \times g_{av}$ for 90 min. The supernate, which was stored at −18°C in 0.2 ml aliquots, was assayed for CAT activity in a medium containing 100 mM Tris–HCl (pH 8.1), 0.5 mM 4,4′-dithio-bis-pyridine (DTBP) or 0.5 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), 0.1 mM acetyl CoA and 3 mM L-carnitine. Release of free sulphydryl groups from acetyl CoA hydrolysis was calculated assuming an extinction coefficient for the pyridone of $\epsilon_{324} = 1.98 \times 10^4$ l . mol^{-1} . cm^{-1} [18] and for the thiophenate anion of $\epsilon_{412} = 1.36 \times 10^4$ l . mol^{-1} . cm^{-1} [19].

2.5. *Protein estimation*

Protein was measured as in [20] using bovine serum albumin as a standard.

2.6. *Chemicals*

DTBP was obtained from the Aldrich Chemical Company, and both DTNB and L-carnitine from the Sigma Chemical Company. BAC was synthesized as in [12]. Both MPF8,167 and MPF15,246 are subject to Patent Applications; their structure and mode of action will be discussed in a subsequent publication. All other chemicals were of the highest purity commercially available from major biochemical suppliers.

3. Results

3.1. *Steady state ATP levels in* T. b. brucei

ATP levels present in *T. b. brucei* incubated in the presence of added D-glucose (20 mM), L-threonine (5 mM and 20 mM) and L-carnitine (10 mM) are shown in table 1. Cells were incubated at 37°C for 1 h although it was found that the steady state concentrations were reached in <10 min. Calibration of the system revealed a linear relationship between luminescence and ATP over 10^{-9}–10^{-14} M.

3.2. *Measurements of ATP synthesis*

The measured rates of ATP synthesis in a trypanosome homogenate incubated at 37°C are shown in table 2. Considerably higher rates were detected in the presence of ouabain and oligomycin indicating the presence of ATPases in the system. The addition of 10 mM L-carnitine to the incubation mixture was accompanied by an increased rate of ATP synthesis ($P < 0.01$, Student's '*t*'-test) and the effect of inhib-

Table 1
Steady state ATP concentrations in *T. b. brucei* (nmol ATP/10^8 cells ± SEM, $n = 5$)

Condition	ATP	
	No carnitine	10 mM L-carnitine
D-Glucose (20 mM)	46.3 ± 3.1	337.2 ± 18.1
L-Threonine (5 mM)	38.8 ± 3.5	81.1 ± 7.6
L-Threonine (20 mM)	26.4 ± 3.8	65.2 ± 6.1

Trypanosomes (10^7/ml) were incubated at 37°C for 1 h in incubation buffer with additions as above before ATP levels were measured as in section 2.2

Table 2
Measured ATP synthesis in *T. b. brucei* homogenates (μmol ATP . h^{-1} . 10^8 $cells^{-1}$ + SEM, n = 5)

(a) Effect of carnitine

Condition	ATP synthesis
Incubation buffer	0.32 ± 0.04
+ L-carnitine (10 mM)	1.57 ± 0.23
+ ouabain (1 mM) + oligomycin (1 mM)	2.21 ± 0.13
+ ATPase inhibitors + L-carnitine (10 mM)	2.53 ± 0.19 ($P < 0.01$)

(b) Effect of inhibitors

Additions		ATP synthesis
MPF15,246	(1 mM)	2.57 ± 0.21
	(10 mM)	2.39 ± 0.20
MPF8, 167	(0.3 mM)	2.21 ± 0.14
	(3 mM)	2.06 ± 0.17
Bromo-acetyl-L-carnitine	(0.1 mM)	2.11 ± 0.23
	(1 mM)	2.03 ± 0.20
	(10 mM)	2.01 ± 0.15

Trypanosome homogenates (5 × 10^8 cells) were incubated in 1 ml incubation buffer at 37°C for 15 min following addition of $Na_2H^{32}PO_4$. Rates of synthesis were measured as in section 2.3. In table 2(b) all incubations were carried out in the presence of ouabain (1 mM), oligomycin (1 mM) and L-carnitine (10 mM)

itors on this increase was also investigated. Both BAC (0.1, 1.0 and 10 mM) and MPF8,167 (0.3 and 3 mM) abolished the carnitine-associated increase in the rate of ATP synthesis and 10 mM MPF15,246, which is metabolized to MPF8,167, also reduced this increase but had little effect at lower concentrations.

3.3. *Measurement of CAT activity*

In view of the possible role of this enzyme in ATP synthesis the possibility of nucleotide control was examined. Table 3 compares the effect of added ADP (1 mM) in the presence of P_i (1 mM) with the inhibitory action of ATP [7]. The effects of MPF15,246, MPF8,167 and BAC on CAT activity are also shown. It is interesting to note the differing effects of the latter 2 compounds, both of which had been found to abolish the carnitine-associated increase in ATP synthesis.

Table 3
Carnitine acetyl transferase activity from *T. b. brucei* (initial rate expressed as % of the control activity equivalent to 90.2 nmol acetyl CoA hydrolysed . min^{-1} . mg cell $protein^{-1}$ ± SEM, n = 4–6)

Conditions	Relative CAT activity
Control	100%
ADP + P_i (1 mM)	172 ± 26%
ATP (1 mM)[a]	50%
MPF15,246 (10 mM)	152 ± 5%
MPF15,246 (1 mM)	113 ± 19%
MPF8,167 (3 mM)	125 ± 7%
MPF8,167 (0.3 mM)	100 ± 8%
Bromo-acetyl-carnitine 10 mM, 1 mM, 0.1 mM	irreversible inhibition

[a] Data from [7]

Enzyme activity measured using 70 μg cell protein/assay as in section 2.4

MPF8,167 showed no inhibition of CAT activity and at 3 mM even showed a slight stimulatory effect. In contrast, all concentrations of BAC showed irreversible inhibition of CAT.

4. Discussion

In the absence of established pathways for fatty acid oxidation in trypanosomes [11] the physiological significance of high carnitine and CAT values in *T. b. brucei* is far from clear. The current work, however, indicates the possibility of a carnitine-associated route for ATP production, perhaps similar to the substrate-level phosphorylation utilizing succinate thiokinase which occurs in the parasitic protozoa *Tritrichomonas foetus* sp. [21].

It is clear (table 1) that the presence of 10 mM L-carnitine increases steady state ATP concentrations in *T. b. brucei* incubated with either glucose or threonine as substrate. That this effect is larger in the presence of glucose is probably a reflection of the limited role of threonine as a respiratory substrate. Although threonine is implicated in pathways of lipid synthesis in *T. b. brucei* [6] it remains doubtful whether this compound is a physiologically important respiratory substrate [22].

The ouabain and oligomycin sensitivity of the assay for ATP synthesis suggests that increased incorporation

of radioactivity into the aqueous layer was indeed due to its incorporation into ATP, and this process was stimulated in the presence of 10 mM L-carnitine (table 2). The inhibition by BAC of the carnitine-associated rise in ATP synthesis is an indication of a role for CAT in this process. The metabolic precursor of MPF8,167 (MPF15,246) showed a much smaller effect than MPF8,167 itself. It is unknown whether this compound can be metabolized by trypanosomes. The measurements of CAT activity revealed two important points. While BAC did inhibit the enzyme, MPF8,167 was inactive; the latter compound, however, was effective at inhibiting the carnitine-associated increase in ATP synthesis. The implication is, therefore, that the site of stimulation by carnitine occurs after the CAT-catalyzed reaction in this pathway of acetate metabolism, perhaps utilizing the high group-transfer potential of one of the products of the reaction (e.g., acetyl-carnitine).

The stimulatory effect of 1 mM ADP and P_i on CAT activity provides circumstantial evidence for the production of ATP by this pathway and is compatible with the finding [7] that ATP itself is a competitive inhibitor of CAT with a K_i of 0.46 mM.

These data suggest a role for the high levels of carnitine and carnitine acetyl transferase in *T. b. brucei*; namely, that these levels are associated with the substrate-level production of ATP and, as such, could provide a useful target for the chemotherapy of the African trypanosomiases.

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