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Purification and characterization of the acetate forming enzyme, acetyl-CoA synthetase (ADP-forming) from the amitochondriate protist, *Giardia lamblia*

Lidya B. Sanchez*, Miklós Müller

The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA

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Abstract ***Giardia lamblia*, an amitochondriate eukaryote, contains acetyl-CoA synthetase (ADP-forming), an enzyme known only from one other eukaryote (*Entamoeba histolytica*) and a few anaerobic prokaryotes. The enzyme has been purified about 350-fold. The activity in the direction of acetate formation was dependent on ADP and inorganic phosphate. The reverse reaction could not be detected. Succinyl-CoA, propionyl-CoA and dADP were utilized with lower efficiency. The enzyme did not utilize AMP plus PP_i thus differs from the broadly distributed acetyl-CoA synthetase (AMP-forming). The enzyme is responsible for acetate production accompanied by ATP generation, thus plays an important role in *G. lamblia* metabolism.**

Key words: Acetyl-CoA synthetase (ADP-forming); Acetate thiokinase; Anaerobic protist; *Giardia lamblia*

1. Introduction

Elimination of acetate as an endproduct of metabolism is observed in many prokaryotes. It is also found in a number of eukaryotic species, almost exclusively in those living in anaerobic or hypoxic habitats [1–3], or exposed temporarily to such conditions [4,5]. This process has been studied in some detail in parasitic protists and helminths but the available information is limited [1,2]. Two different pathways have been recognized. In both cases acetate is derived from acetyl-CoA and the energy of the thioester bond is conserved by substrate level phosphorylation. In one of these, characterized first from the anaerobic protist *Entamoeba histolytica* [6], the process is catalyzed by a single enzyme, acetyl-CoA synthetase (ADP forming), or acetate thiokinase (EC 6.2.1.13):

$$\text{acetyl-CoA} + \text{ADP} + P_i \leftrightarrow \text{acetate} + \text{ATP} + \text{CoA-SH} \quad (1)$$

The other pathway comprises two reactions that separate the liberation of acetate by acetate:succinate CoA-transferase (EC 2.3.1.99) and the conservation of the energy of the thioester bond by succinyl-CoA synthetase (EC 6.2.1.4):

$$\text{acetyl-CoA} + \text{succinate} \leftrightarrow \text{acetate} + \text{succinyl-CoA-SH} \quad (2)$$

$$\text{succinyl-CoA} + \text{ADP} + P_i \leftrightarrow \text{acetate} + \text{ATP} + \text{CoA-SH} \quad (3)$$

$$\text{acetyl-CoA} + \text{ADP} + P_i \xleftrightarrow{\text{succinate}} \text{acetate} + \text{ATP} + \text{CoA-SH} \quad (2+3)$$

Reaction (1) and the enzyme responsible for it have been detected only in a few organisms so far [3,6–8]. The two-step process has been documented from a greater diversity of organisms [3,9,10], but also remains relatively unexplored. A two-step pathway of the formation of acetate from acetyl-CoA with the overall reaction of that of acetyl-CoA synthetase, proceeding via free acetyl-phosphate, is broadly distributed in eubacteria [11], but is absent from eukaryotic organisms with one exception reported [12].

An enzyme present in many eubacteria and eukaryotes is acetyl-CoA synthetase (AMP-forming) (EC 6.2.1.1):

$$\text{acetyl-CoA} + \text{AMP} + PP_i \leftrightarrow \text{acetate} + \text{ATP} + \text{CoA} \quad (4)$$

The biological role of this enzyme is generally not in the formation of acetate but in its utilization through activation to acetyl-CoA [11]. Knowledge of the characteristics and taxonomic distribution of the various modes of acetate formation, an important process of energy conservation, could provide clues to a reconstruction of the phylogenetic history of eukaryotic energy metabolism, a topic currently with more speculation than clear insights. Studies on representatives of early branches of eukaryotic evolution should be especially revealing [13].

We report here the purification and characterization of the acetyl-CoA synthetase (ADP-forming) of the enteric parasitic protist *Giardia lamblia*, which belongs to one of the earliest branches of the eukaryotic evolutionary tree [14,15]. This amitochondriate organism has a fermentative metabolism both in the absence and in the presence of oxygen [3,16–18]. Our results confirm the detection of this activity in cell homogenates of *G. lamblia* [19]. They support the notion that the amitochondriate protists without metabolic compartmentation are characterized by the presence of this one-step process of acetate formation from acetyl-CoA [3,13].

2. Materials and methods

2.1. Parasites

Giardia lamblia, (strain WB, ATCC 30957, clone 6) trophozoites, kindly given to us by Dr. Steven Aley (University of California, San Diego, CA), were grown axenically at 37°C in Diamond's TYI-S-33 medium [20].

2.2. Preparation of parasite extracts

Logarithmic phase cultures were chilled on ice for 20 min and harvested by centrifugation at 800 rpm for 7 min. The cell pellet was

*Corresponding author. Fax: (1) (212) 327-7974.
E-mail: sanchel@rockvax.rockefeller.edu

Abbreviations: CoA-SH, coenzyme A; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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washed twice with 250 mM sucrose containing 5 $\mu g \cdot ml^{-1}$ leupeptin and resuspended in 25 mM Tris-HCl pH 7.5, containing 5 $\mu g \cdot ml^{-1}$ leupeptin. Cells were disrupted in a Potter homogenizer with Teflon pestle, and the homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant fraction was then centrifuged at 26,000 rpm for 60 min and 20% glycerol was added to the final supernatant, which was aliquoted and stored at −20°C until used.

2.3. Determination of acetyl-CoA synthetase activity

Acetyl-CoA synthetase activity was determined in the direction of acetate formation by following the release of CoA-SH from acetyl-CoA with Ellman's thiol reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [21]. The standard assay mixture contained 0.05 mM acetyl-CoA, 2 mM ADP, 40 mM potassium phosphate, 10 mM $MgCl_2$ in 50 mM Tris-HCl, pH 7.5, and 0.1 mM DTNB. Due to the instability of the enzyme preparations, for kinetic determinations 20% glycerol and 1 $\mu g \cdot ml^{-1}$ ovalbumin were included in the reaction mixture. The reaction was started with the addition of the enzyme sample and the increase in absorbance due to the formation of thiophenolate was monitored at 412 nm ($\varepsilon_{412} = 13600\ M^{-1} \cdot cm^{-1}$). One unit of enzyme activity is defined as 1 μmol of CoA-SH released per minute.

For the reaction in the direction of acetyl-CoA formation three different assays were tested. (1) Coupling the reaction to the oxidation of NADH via pyruvate kinase and lactate dehydrogenase [7]. The assay mixture contained: 2 mM ATP, 10 mM sodium acetate, 0.5 mM CoA, 10 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 0.3 mM NADH, 4 U pyruvate kinase and 25 U lactate dehydrogenase in 100 mM Tris-HCl, pH 7.5. (2) Detection of acetylhydroxamate formation in the presence of 5 mM sodium acetate, 5 mM ATP, 0.5 mM CoA, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5 and 0.3 M neutralized hydroxylamine. The reaction was stopped by the addition of 1 volume of 10% (w/v) $FeCl_3 \cdot 6H_2O$/0.7 M HCl and the hydroxamate formed was determined at 505 nm [11]. (3) Determining the unreacted CoA-SH with DTNB in a reaction mixture containing 10 mM $MgCl_2$ 5 mM sodium acetate, 5 mM ATP and 0.5 mM CoA in 50 mM Tris-HCl, pH 7.5. Yeast acetyl-CoA synthetase (Sigma) was used as control for these reactions.

2.4. Purification of acetyl-CoA synthetase activity

Acetyl-CoA synthetase activity was purified by polyethylene glycol precipitation followed by three successive adsorption steps. All steps were carried out at 0–4°C and all the buffers contained 20% glycerol. 18% (v/v) polyethylene 4000 was added to the crude extract, and then incubated for 30 min. The precipitated material, containing the enzyme activity, was collected by centrifugation at 100,000 × *g* for 30 min and resuspended in 0.5 volume of 20 mM MES-Tris buffer/20% glycerol, pH 7.0 (Buffer A). The subsequent steps were carried out by stirring various chromatographic adsorbents in batches into the preparation. After adsorption for 5 min, the material was centrifuged at 700 rpm for 2 min. The supernatant was removed and the resin was washed five times, in the same way, with one volume of the washing buffer and five times with one volume of washing buffer containing either KCl or NaCl, as specified for each resin. In the first step 2 volumes of Q-Sepharose (Pharmacia), previously equilibrated with Buffer A, were added. Q-Sepharose was washed first with Buffer A and then with the same buffer containing 0.2 M NaCl. To the fractions containing most of the activity was added 2 volume of CM-Sepharose (Pharmacia), pre-equilibrated with Buffer A and after 5 min the resin was washed under the same conditions as for Q-Sepharose. The fractions with the highest activity were pooled, desalted and the buffer changed to 20 mM Tris-HCl, pH 7.5/20% glycerol (Buffer B) by three rounds of concentration-dilution in a stirred pressure cell with YM30 membrane (Amicon). The resulting preparation was adsorbed into 2 volumes of Matrex gel red A dye (Amicon), equilibrated with Buffer B, the resin washed with 5 volumes of Buffer B and the activity eluted with 0.2 M KCl in Buffer B. This pooled fraction was desalted and concentrated through a YM30 membrane and stored at −20°C until used.

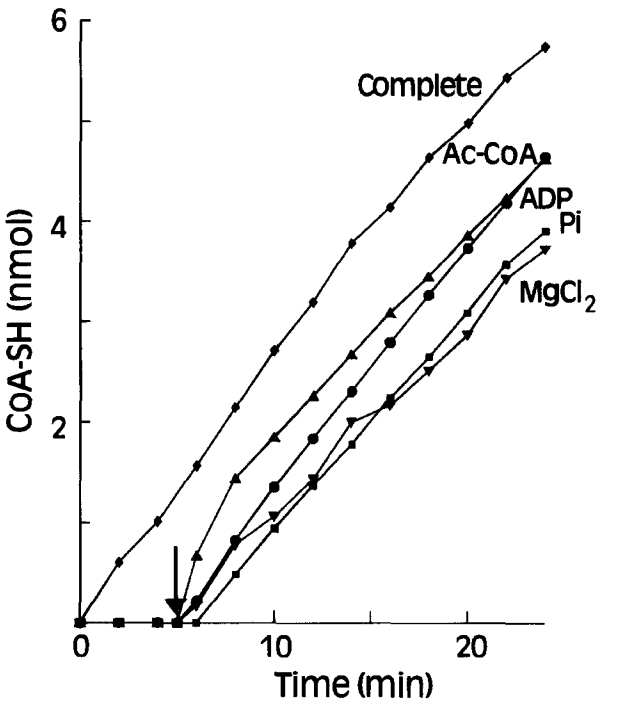

Fig. 1. Requirements for the *Giardia lamblia* acetyl-CoA synthetase activity. Release of CoA-SH was monitored in the presence of DTNB in the complete reaction mixture (◆) described in section 2; and in mixtures from which one of the following substrates was omitted: acetyl-CoA (▲); ADP (●); inorganic phosphate (■) or $MgCl_2$ (▼). At 5 min (arrow) the missing component was added at the same concentration as for the complete reaction. The reaction was followed spectrophotometrically at 412 nm.

2.5. Other methods

Protein was determined by the method of Bradford (Bio-Rad protein reagent) with bovine serum albumin as the standard.

3. Results

3.1. Enzyme activity and protein purification

Acetyl-CoA synthetase activity was monitored with the assay in the direction of acetate formation. The specific activity of whole homogenates of *G. lamblia* was 250–500 $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$. After centrifugation of the homogenate about 70–75% of the activity was found in the nonsedimentable frac-

Table 1
Purification of *Giardia lamblia* acetyl-CoA thiokinase (ADP-forming)

Procedure	Total protein (μg)	Total activity (U)	Yield (%)	Specific activity[a]	Purification (-fold)
Crude extract	30,984	14.38	100	0.5	–
PEG precipitation	9,437	8.82	61	1.3	2.6
Q-Sepharose[b]	4,615	9.67	68	2.2	4.4
CM-Sepharose	145	2.59	18	15.6	31.0
Matrex gel Red A	6	1.15	8	178.0	356.0

[a] μmol of CoA-SH release per minute per milligram of protein.
[b] Activity did not bind but this step removed about 50% of the protein from the preparation.

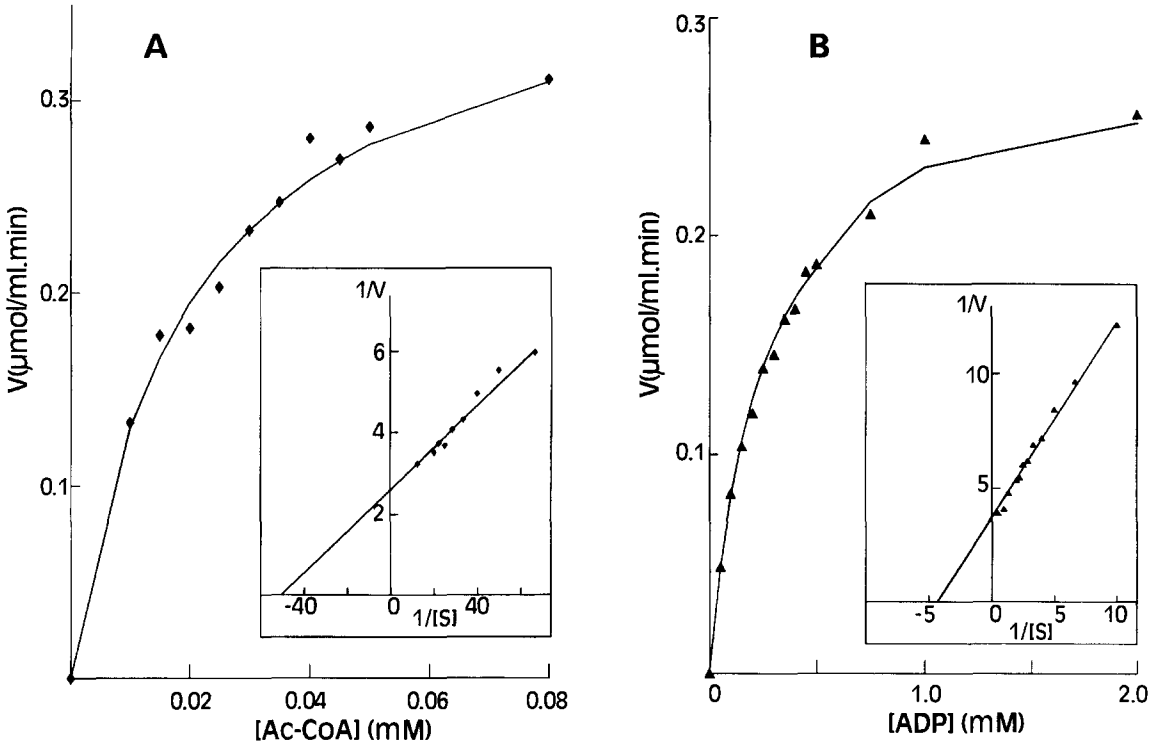

Fig. 2. Dependence of *G. lamblia* acetyl-CoA synthetase activity on the concentration of acetyl-CoA (A) and ADP (B). Activity measured in the direction of acetate formation. The insets show double reciprocal plots of the rates versus the corresponding substrate concentrations.

tion. The remaining 25–30% of the activity could be detected in the pellet after its resuspension and sonication, but had a much lower specific activity. Starting from the nonsedimentable fraction the enzyme was purified 350-fold (Table 1). Acetyl-CoA synthetase activity was precipitated with polyethylene. Q-Sepharose did not bind the enzyme but by removing other proteins, it enriched the preparation about 2-fold. The first two fractions, containing most of the acetyl-CoA synthetase activity were adsorbed to CM- Sepharose. Unbound protein was removed by washing and then the activity eluted with 0.2 M NaCl. Fractions containing most of the activity were combined and desalted by ultrafiltration. Finally the enzyme was affinity purified by adsorption on Matrex gel red A dye and elution with 0.2 M KCl. The final yield of the purified activity was about 8%. Although a high degree of purification was achieved, the preparation was not yet homogeneous, as detected by SDS-PAGE and silver staining (not shown).

The enzyme is unstable, leading to major losses during and after purification. Crude extracts, in Buffer B containing glycerol, retained their activity for several months at −20°C, but completely lost activity within a week at 4°C. Purified preparations were even less stable and could not be stored at −20°C without a considerable loss of activity (more than 70–80% in 5 days). Addition of $MgCl_2$, EDTA, ADP to the buffer or keeping the sample under nitrogen did not increase the stability of the enzyme. The low yield of enzyme activity and the necessity to use fresh preparations throughout the study presented significant difficulties.

3.2. Requirements for the acetyl-CoA synthetase reaction

Fig. 1 shows the requirements of the purified acetyl-CoA synthetase for acetyl-CoA, ADP, P_i and Mg^{2+}. When all of the required substrates are present in the reaction mixture, CoA-SH is liberated from acetyl-CoA. That all these components are required is shown by the lack of reaction when any one of them was omitted from the mixture, and its commencement soon after the missing substrate is added (arrow). Controls without acetyl-CoA showed no liberation of free SH groups. When the phosphoryl group acceptor, ADP, was replaced with AMP and the phosphate donor, P_i, with PP_i or ATP, only a negligible release of CoA-SH (1–2% of that measured in the presence of ADP plus P_i) was detected. This reveals the ADP specificity of the *G. lamblia* enzyme.

The reaction exhibited a narrow pH optimum in Tris-HCl buffer, pH 6.0–7.5, being most active at pH 7.2. Similar behavior was observed in acetate/cacodylate/borate buffer (not shown).

3.3. Kinetics and substrate specificity

The rate of the reaction at different concentrations of acetyl-CoA, ADP (Fig. 2), inorganic phosphate and $MgCl_2$ (not shown) followed Michaelis-Menten kinetics, giving linear double reciprocal plots. Calculated K_m values were 0.02 mM for acetyl-CoA, 0.23 mM for ADP, 1.59 mM for phosphate and 0.59 mM for $MgCl_2$.

To characterize the specificity of this enzyme, other nucleotide diphosphates, dADP and different acyl-CoA esters were tested as substrates. The *G. lamblia* enzyme used ADP preferentially. Activity with dADP was 40% of that measured with ADP and the enzyme showed lower affinity (K_m = 1.09 mM). CDP, GDP or UDP did not support any CoA-SH release. The relative lower K_m and higher V_{max} values for acetyl-CoA indicates

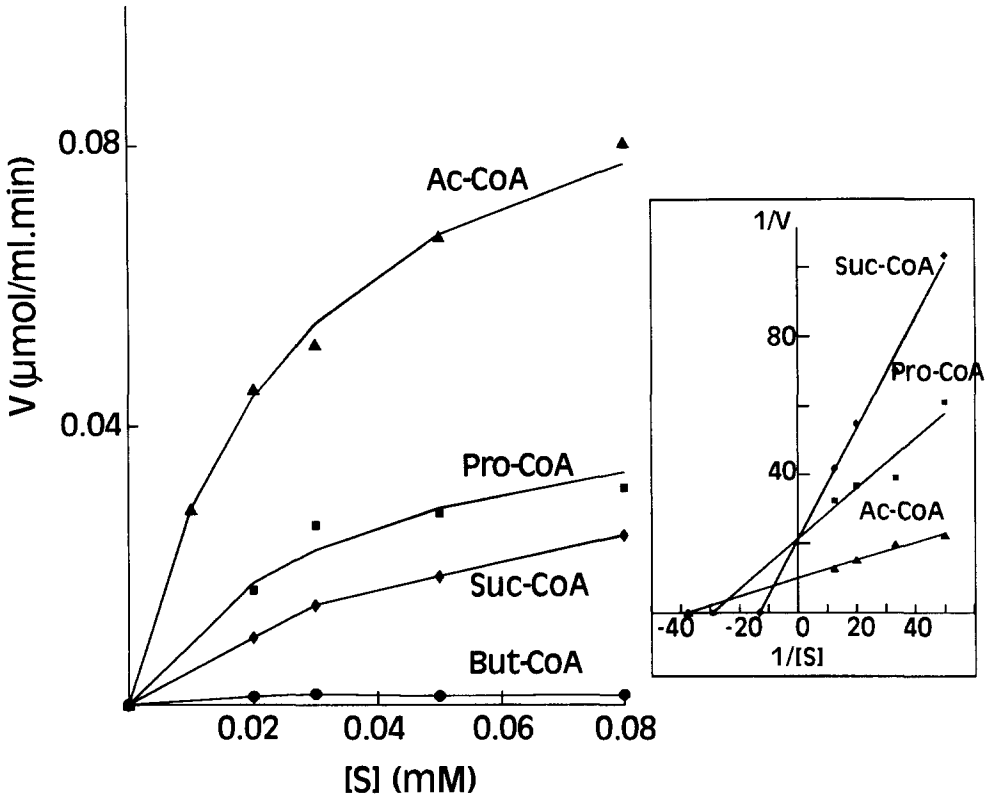

Fig. 3. Acyl-CoA ester specificity of *G. lamblia* acetyl-CoA synthetase. CoA-SH released from acetyl-CoA (▲), *n*-propionyl-CoA (■), succinyl-CoA (◆) and *n*-butyryl-CoA (●) was determined at the indicated substate concentrations. The inset shows the double reciprocal plots of the rates versus the corresponding substrate concentration.

that this is the preferred substrate for the enzyme (Fig. 3). *n*-Propionyl-CoA and succinyl-CoA were also utilized with lower efficiency (39% and 30%, respectively) but *n*-butyryl-CoA was not. When acetyl-CoA and succinyl-CoA were present together the activity observed was lower than that observed with acetyl-CoA only (not shown), indicating the presence of one acyl-CoA synthetase species.

The effect of mono- and divalent cations in the form of their chlorides was tested in the standard assay. Divalent cations are required for the reaction. Mn^{2+} and Co^{2+} supported acetyl-CoA synthetase activity as well as Mg^{2+}, whereas Ca^{2+} was less effective (15% of the activity with Mg^{2+}). Monovalent cations (K^+, Na^+, Li^+ and NH_4^+) had no effect.

3.4. Failure to detect the reverse reaction

Using three different assays, generally used to measure other acetyl-CoA synthetases, activity in the direction of acetyl-CoA formation could not be detected. The reason for this observation is not clear and a more detailed exploration is required before a more conclusive statement can be made.

4. Discussion

The data presented show that *G. lamblia* is among the few microorganisms known to contain an ADP-dependent acetyl-CoA synthetase [6–8]. These results confirm an earlier report based on the study of cell free extracts [19]. The properties of the enzyme correspond to its assumed role in the formation of acetate, a known end product of the energy metabolism of this organism [16,19]. It should be noted that the ADP-specificity of the enzyme is indicative of the physiological direction of the reaction catalyzed by it. ATP formation by substrate level phosphorylation is usually ADP-dependent while processes liberating PP_i from a nucleotide triphosphate are generally activating a metabolite for synthetic reactions [11].

The activity observed in the homogenates is sufficient to account for the in vivo acetate production of the organism [19]. The properties of the enzyme indicate that it is one of the main actors in ATP generation in *G. lamblia*. It should be noted, however, that this organism also utilizes arginine and possibly other amino acids as energy substrates, and eliminates also alanine and ethanol [16] as major metabolic end products. The proportion of the end products varies under different conditions [16,17] indicating branched pathways of energy metabolism. The relative importance of the various energy substrates and the regulation of the metabolite flow through the various branches remains to be elucidated.

The enzyme is similar in its characteristics to the few known ADP-forming acetyl-CoA synthetases [6–8]. Its acyl-CoA specificity was similar to that of the enzyme from the eubacterium *Selenomonas ruminantium* [8]. In contrast the *E. histolytica* enzyme has no succinyl-CoA synthetase activity [6]. The enzymes of the other species were shown to act also in the direction of acyl-CoA synthesis. The apparent irreversibility of the *G. lamblia* enzyme warrants further exploration.

The only other eukaryotic species, where this enzyme has

been detected, *E. histolytica* [6] is also an enteric parasitic protist, without metabolic compartmentation [3]. These two organisms, however, belong to two independent lineages, which separated at different times from the main branch of eukaryotic evolution [14]. Both are enteric parasites raising the possibility that the presence of this enzyme, as well as a number of other metabolic characteristic shared between them [3] do not represent relics of early eukaryotic evolution as has been suggested [13] but are connected with their parasitic mode of life. This intriguing question can be resolved only by a broader taxonomic sampling, encompassing both free-living and parasitic relatives of these organisms [3]. The detection of this enzyme in organisms belonging to all three domains of life might indicate that further search will disclose its broader distribution.

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