Aspartate Transcarbamylase from *Leishmania donovani*

A DISCRETE, NONREGULATORY ENZYME AS A POTENTIAL CHEMOTHERAPEUTIC SITE*  

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Leishmania donovani is a protozoal pathogen that belongs to the kinetoplastida order. Unlike in other eucaryotic systems, the first three enzymes of the *de novo* pyrimidine biosynthetic pathway are not components of a multifunctional protein system. The three enzyme activities in the crude extract were separated on a Sephadex S-200 column.  

Aspartate carbamoyltransferase (EC 2.1.3.2) has been purified to apparent homogeneity. The enzyme has an approximate molecular weight of 135,000 and seems to be a tetramer of equivalent subunits of molecular weight 35,000. The enzyme shows strictly hyperbolic kinetics with both the substrates under a variety of conditions and is not inhibited by nucleotide phosphates. \( K_m \) for carbamyl phosphate is 3.1 \( \times 10^{-5} \) M and for aspartate is 7.6 \( \times 10^{-5} \) M. Apparently, the enzyme has no regulatory role in pyrimidine biosynthesis.  

\( N\)-(Phosphonacetyl)-L-aspartic acid is a powerful competitive inhibitor (\( K_i = 5 \times 10^{-7} \) M) for this enzyme with carbamyl phosphate as substrate. This inhibitor completely inhibits the growth of the vector form of organism at 60 \( \mu \)M and significantly affects the growth of the pathogenic form in a macrophage assay system. The potency of the inhibitor is comparable with allopurinol which is undergoing human clinical trial as an antileishmanial drug.

The enzymatic and genomic organization of pyrimidine biosynthetic pathway in eucaryotes presents some unusual features of considerable interest for comparative biochemistry and molecular biology (1). The six enzyme activities required for the *de novo* biosynthesis of the pyrimidine ring are discrete proteins in procaryotic systems. In contrast, in higher eucaryotes, some of these enzyme activities are expressed in a single polypeptide chain. In mammals, as well as in *Drosophila*, the first three enzymes of the pathway, namely carbamoyl-phosphate synthetase (ammonia) (EC 6.3.4.16), aspartate carbamoyltransferase (EC 2.1.3.2), and dihydroorotase (EC 3.5.2.3) are encoded by a single structural gene that produced a single polypeptide of trimeric quaternary structure with all three enzymatic activities (1-3). The yeast and the *Neurospora* also express a multienzyme protein, but this has activity for only the first two enzymes of the pathway (4). Dihydroorotase is a discreet enzyme for these organisms. The situation in other types of lower eucaryotes that include the unicellular prokaryotic systems remains essentially unexplored.  

Leishmania donovani is an important member of the kinetoplastida group of protozoal parasites. The organism has a digenic life cycle: a flagellated promastigote form in the sand fly vector and a nonflagellated amastigote or pathogenic form in macrophage systems of hosts. The organism is the causative agent for kala azar, a lethal form of visceral leishmaniasis, that is widely prevalent in many parts of the tropical world (5, 6). The kinetoplastida, including *L. donovani*, are unable to synthesize purine *de novo* and depend for their purine requirement on preformed purines of the host and its own salvage pathway (7, 8). In contrast, the kinetoplastida appears to have the enzymatic machinery necessary to synthesize pyrimidine *de novo*. All six enzymes of the pyrimidine pathway have been detected in the promastigote form of *L. mexicana*, in the trypomastigote form of *Trypanosoma brucei* (9-11). Isotopic studies with *L. donovani* promastigotes indicate the presence of both the *de novo* and the salvage pathway in this organism (12). However, apart from these initial works, very few systematic studies have been reported on the characterization of the individual enzymes of the pyrimidine pathway in kinetoplastida. Important questions of possible existence of multifunctional single polypeptide enzyme systems, enzymatic regulatory sites, and potential chemotherapeutic sites etc. all remain unexplored at the moment. In this paper, we report that, in the cultural or the promastigote form of *Leishmania donovani*, the first three enzymes of the pyrimidine biosynthetic pathway remain as three discrete proteins with separable enzyme activities. Further, extensive purification and characterization of aspartate transcarbamylase show that unlike in many bacterial systems, this enzyme does not have any obvious regulatory role in this biosynthetic pathway. Finally, \( N\)-(phosphonacetyl)-L-aspartic acid, a transition state analogue for this enzymatic reaction, has a powerful growth-inhibitory effect on both the promastigote form and on the model screening system for the pathogenic amastigote form. This inhibition by PALA is fairly comparable with allopurinol that is undergoing clinical trial for visceral leishmaniasis at present (13).

MATERIALS AND METHODS AND RESULTS

Separation of Carbamyl Phosphate Synthetase, Aspartate Transcarbamylase and Dihydroorotase Activities—In crude
Aspartate Transcarbamylase from L. donovani

extracts of L. donovani, carbamyl phosphate synthetase activity was found to be very unstable. The activity could be stabilized for subsequent operations only in the presence of glycerol and dimethyl sulfoxide. In a typical experiment, washed pelleted cell (3 g) was suspended in 3.0 ml of glass-distilled water containing 1 mM DTT, 5 mM benzamidine, 0.1 mM PMSF, 0.75 mg of soybean trypsin inhibitor and allowed to swell for 15 min. 1.75 ml of dimethyl sulfoxide/glycerol mixture (6:1) was added to the swelled cells and homogenized in a Potter-Elvehjem homogenizer. The extract was adjusted to 50 mM Tris-HCl buffer, pH 7.2, and centrifuged at 18,000 \(\times g\) for 30 min. One ml of the supernatant was then applied on a Sephacryl S-200 column (57 X 1.25 cm), previously equilibrated with 50 mM Tris-HCl buffer, pH 7.5, 1 mM DTT, 30% dimethyl sulfoxide, and 5% glycerol. The column was eluted also with 50 mM Tris-HCl buffer containing 1 mM DTT, 5 mM benzamidine, 0.1 mM PMSF, 0.75 mg of soybean trypsin inhibitor and allowed to swell for 15 min. 1.75 ml of dimethyl sulfoxide/glycerol mixture (6:1) was added to the swelled cells and homogenized in a Potter-Elvehjem homogenizer. The extract was adjusted to 50 mM Tris-HCl buffer, pH 7.2, and centrifuged at 18,000 \(\times g\) for 30 min. One ml of the supernatant was then applied on a Sephacryl S-200 column (57 X 1.25 cm), previously equilibrated with 50 mM Tris-HCl buffer, pH 7.5, 1 mM DTT, 30% dimethyl sulfoxide, and 5% glycerol. The column was eluted also with 50 mM Tris-HCl buffer containing 1 mM DTT, 30% dimethyl sulfoxide, and 5% glycerol at a flow rate of 6 ml/h.

The elution profile is shown in Fig. 1. It is evident that the three enzymatic activities were separated as distinct activity peaks on this column. In a separate experiment, using a partially purified enzyme fraction, we could demonstrate the separation of aspartate transcarbamylase and dihydroorotase activities on a Sephadex G-100 column (see Miniprint Section). In this case, however, we could not detect any activity for carbamyl phosphate synthetase which was presumably lost during processing.

Purification and Characterization of Aspartate Transcarbamylase—Results of a typical purification procedure are summarized in Table I. The approximate molecular weight of the purified homogenous protein was calculated to be 135,000, and analysis under denaturing conditions showed it to consist of a single subunit of molecular weight 35,000. The enzyme failed to show any sigmoidal kinetics under a variety of conditions and was not significantly inhibited or activated by a large number of related metabolites. PALA was found to be a strong inhibitor of pure aspartate transcarbamylase with a \(K_i\) of 0.5 \(\mu\)M. Experimental details of these results are presented in the Miniprint.

Effect of PALA on Growth of L. donovani Promastigotes—

**TABLE I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Initial Specific Activity (units/mg)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Crude</td>
<td>451</td>
<td>0.035</td>
<td>1.0</td>
</tr>
<tr>
<td>(ii) Protamine sulfate step</td>
<td>276</td>
<td>0.655</td>
<td>1.6</td>
</tr>
<tr>
<td>(iii) First ammonium sulfate step</td>
<td>82</td>
<td>0.179</td>
<td>5.1</td>
</tr>
<tr>
<td>(iv) Heat treatment</td>
<td>25.5</td>
<td>1.17</td>
<td>13.0</td>
</tr>
<tr>
<td>(v) Second ammonium sulfate step</td>
<td>14.4</td>
<td>0.701</td>
<td>20.0</td>
</tr>
<tr>
<td>(vi) Sephadex G-100 column</td>
<td>2.6</td>
<td>1.96</td>
<td>56.0</td>
</tr>
<tr>
<td>(vii) DEAE-cellulose column</td>
<td>0.12</td>
<td>9.16</td>
<td>261.0</td>
</tr>
</tbody>
</table>

**Fig. 1.** Profile of carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activities, eluted from Sephacryl S-200 column. The crude extract containing 12 mg of protein in 1 ml of crude extract was applied to a column (57 X 1.25 cm), previously equilibrated with 50 mM Tris-HCl buffer, pH 7.5, 1 mM DTT, 30% dimethyl sulfoxide, 5% glycerol. Elution rate was 6 ml/h and 1.5-ml fractions were collected. Carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activity are represented by □, ■, ■, ■, and ▲, ▲, respectively. The void volume is indicated.

![Inhibition of growth of L. donovani](image_url)

**Fig. 2.** Inhibition of growth of L. donovani in the presence of PALA. The control growth curve in the absence of PALA is represented by ■, ■, ▲, ▲, and ○—○ indicate growth pattern in the presence of 40, 60, and 150 \(\mu\)M PALA, respectively.

PALA has been found to be a potent inhibitor for growth of several mammalian cell lines including transformed cells (14, 15). PALA either alone or in combination with other anticancer drugs is undergoing screening as a possible anti-tumor agent (16, 17). In view of the strong inhibition of PALA on L. donovani aspartate transcarbamylase, its possible inhibitory effect on the growth of L. donovani promastigotes was checked. Fig. 2 shows that PALA is a fairly powerful inhibitor of growth for the organism, and the minimum inhibitory concentration was calculated to be approximately 60 \(\mu\)M. The 50% effective dose is less than 20 \(\mu\)M (data not shown) and is quite comparable in its effect with allopurinol and other pyrazolopyrimidine analogues that are being developed as possible chemotherapeutic agents against the pathogenic kinetoplastida (13, 18). We could further demonstrate that addition of uracil or uridine (100 \(\mu\)M) in the growth medium
could substantially protect the organism (nearly 50%) against inhibition by PALA (data not shown).

**Effect of PALA on the Growth of the Pathogenic Form of L. donovani**—PALA was found to have a definite inhibitory effect on the multiplication of the pathogenic or amastigote form, in the in vitro macrophage assay system. This is evident from Table II. PALA (30 μM) could significantly retard the growth of Leishmania amastigotes when assayed in the hamster peritoneal macrophage system. The extent of inhibition observed with pentamidine which was used as the control drug agrees well with the recently reported value for this drug in the same assay system (19). Increasing the concentration of PALA to 50 μM did not result in further inhibition of growth.

**DISCUSSION**

Resolution of carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotatase activities on a Sephadex G-200 column (Fig. 1) and of the latter two activities on Sephadex G-100 column (Fig. S1, Miniprint) clearly show that in L. donovani the three enzymes are not components of a multifunctional protein system. Partial separation of the three activities had earlier been achieved by density gradient technique in *Toxoplasma gondii* (11), a parasitic protozoa belonging to a different family. Apparently, in all protozoal systems, the three enzymes are expressed as three distinct proteins, and gene fusion for these activities may have started at the level of yeast and *Neurospora* (1). Interestingly, a multifunctional protein system has recently been detected in *Leishmania tropica* (20, 21). In this case, an overproduction of a bifunctional thymidylate synthetase dihydrofolate reductase protein takes place because of gene amplification when the organism is gradually exposed to higher concentrations of methotrexate.

*Leishmania* aspartate transcarbamylase is probably a tetramer of four identical subunits (Figs. S1B and S2, Miniprint). The enzyme failed to show any regulatory property or cooperative kinetic phenomenon under a variety of conditions. Apparently, the flux of pyrimidine biosynthetic pathway is regulated at some other enzymatic step of this pathway. The quaternary structure of the enzyme, absence of a second subunit, and general lack of sensitivity to nucleotides suggest some resemblance with the enzymes from *Streptococcus faecalis* or *Bacillus subtilis* (22).

The moderately strong growth inhibitory property of PALA for both the vector (Fig. 2) and the host pathogenic form (Table II) may be of some chemotherapeutic value. Considering the extremely high affinity of PALA for leishmanial aspartate transcarbamylase, the extent of inhibition or the concentration needed for complete inhibition of growth is not remarkable. Inefficient uptake of PALA to build up an effective cellular concentration may be a possible cause for this discrepancy. In any case, when compared to allopurinol (18, 23) which is already undergoing clinical trial with some success (24), the concentration of PALA to get the desired effects is fairly encouraging. In view of these results, the possibility of combination therapy where the purine salvage pathway and the *de novo* pyrimidine pathway are simultaneously inhibited should be explored. This is particularly relevant in the context of increasing reports of resistance to the treatment of pentavalent antimonials in the case of kala azar (24).

Our present study with aspartate transcarbamylase from *L. donovani* indicates that sustained and intensive work on the enzymes of this pathway will be useful in several directions. PALA can possibly be exploited as a probe for studying the phenomenon of gene amplification and drug resistance as it is being done in mammalian systems (25). Its potential as a chemotherapeutic agent, either alone or in combination, after entrapment in liposomes or in suitable carriers should be further explored. In experimental models of leishmaniasis, passive targeting to liver macrophages of pentavalent antimonia encapsulated in liposomes has already shown considerable promise (26). Extensive knowledge on the regulatory and other properties of the *de novo* pyrimidine pathway is expected to contribute significantly to the comparative biochemistry of protozoal systems and to development of new avenues for chemotherapy.

**ACKNOWLEDGMENTS**—We thank Pannalal Dutta, Dr. Ananta Ghosh, and Dr. D. K. Ghosh for helping us with biological experiments and Dr. Dwijen Sarkar for helpful suggestions.

**REFERENCES**

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Additional references are found below.

MATERIALS AND METHODS

All the biochemicals, unless otherwise mentioned, were purchased from Sigma Chemical Co. (U.S.A.). New Zealand White rabbit was obtained as a gift from Dr. R. K. Sandhya at the Department of Biotechnology, Indian Institute of Technology, New Delhi. All other chemicals were of reagent grade or of the highest available purity.

The precipitated cells were collected by centrifugation at 10,000 g for 30 min and then resuspended in 20 mM TRIS-HCl pH 7.4 buffer containing 0.5 mM EDTA. The volume of the suspension was adjusted to 40 ml with 20 mM EDTA.

The solution after this second ammonium sulfate treatment was passed through a column of Sephadex G-100 (10 cm × 63 cm) previously equilibrated with buffer. The fractions containing the enzyme were pooled and concentrated by dialysis against a buffer A (200 mM NaCl, 100 mM TRIS-HCl pH 7.4, 1 mM EDTA) using a stirring magnet. The enzyme was then dialyzed against the same buffer but without NaCl, and then lyophilized.

3. The precipitated cells were centrifuged at 15,000 g for 30 min and the supernatant solution was used as crude extract. This fraction was designated as the first ammonium sulfate fraction. This fraction was then dialyzed against 20 mM TRIS-HCl pH 7.4 and the enzyme was then dialyzed against the same buffer but without sodium chloride using a stirring magnet. This fraction was designated as the second ammonium sulfate fraction. The enzyme was then dialyzed against the same buffer but without sodium chloride until equilibration was achieved.

4. The precipitated cells were then centrifuged at 15,000 g for 30 min and the supernatant solution was used as crude extract. This fraction was designated as the first ammonium sulfate fraction. This fraction was then dialyzed against 20 mM TRIS-HCl pH 7.4 and the enzyme was then dialyzed against the same buffer but without sodium chloride using a stirring magnet. This fraction was designated as the second ammonium sulfate fraction. The enzyme was then dialyzed against the same buffer but without sodium chloride until equilibration was achieved.

REFERENCES

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Figs. 1A & 1B: Polyacrylamide gel electrophoresis and sodium-dodecyl sulphate-polyacrylamide gel electrophoresis of ATCase: First tube (Fig. 1A) polyacrylamide gel electrophoresis, second tube (Fig. 1B) sodium-dodecyl sulphate-polyacrylamide gel electrophoresis. The amount of protein in first tube was 40 μg and in second tube was 0.1 mg. The stain used was Coomassie brilliant blue. The migration was from top (negative) to bottom (anode). In first tube (Fig. 1A) lower band represents dye front.

Fig. 2: Determination of molecular weight of ATCase by gel filtration on a calibrated sephadex G-200 column: The void volume of the column was determined by running protein and Vo is the void volume of the column.

Fig. 3: Determination of subunit molecular weight of ATCase by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Fig. 4: Effect of L-aspartate on inhibition of ATCase by PALA: Enzyme activity was assayed as described in Materials and Methods section. Appropriate amount of the purified enzyme was used per assay with fixed concentration of carbamyl phosphate (1 mM). The concentration of PALA were: zero; 0.01 mM; 0.02 mM.

Fig. 5: Competition of PALA with carbamyl phosphate for ATCase: Enzyme activity was assayed as described in Materials and Methods section. Fixed concentration of L-aspartate (10 mM) was used in each assay. Appropriate amount of the purified ATCase was used for the assay. The concentrations of PALA were: zero; 0.01 mM; 0.02 mM.
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Fig. 6: Profile of ATCase, TIMase activities and protein eluted from Sephadex G-100 column. The ammonium sulphate precipitate containing 22 mg of protein in 0.5 ml of buffer B was applied to a column. The elution rate was 5 ml/hr and 1 ml fractions were collected. Protein content was estimated by the trichloroacetic acid method. Activities of ATCase and TIMase are represented by (O) and (△), respectively.