# Pyrimidine Biosynthesis in Parasitic Protozoa: Purification of a Monofunctional Dihydroorotase from *Plasmodium berghei* and *Crithidia fasciculata*†

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ABSTRACT: Dihydroorotase (DHOase) catalyzes the reversible cyclization of *N*-carbamoyl-L-aspartate (L-CA) to L-5,6-dihydroorotate (L-DHO), which is the third enzyme in de novo pyrimidine biosynthesis. The enzyme was purified from two parasitic protozoa, *Crithidia fasciculata* (about 16000-fold) and *Plasmodium berghei* (about 790-fold). The *C. fasciculata* enzyme had a native molecular weight ($M_r$) of 42000 ± 5000, determined by gel filtration chromatography, and showed a single detectable protein band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with $M_r$ 44000 ± 3000. The DHOase from *P. berghei* had a native molecular weight of 40000 ± 4000 and a subunit molecular weight on SDS–PAGE of 38000 ± 3000. The DHOase from both parasites, in contrast to the mammalian enzyme which resides on a trifunctional protein of the first two enzymes of the pathway, carbamoyl-phosphate synthase and aspartate transcarbamylase, is monomeric and has no oligomeric structure as studied by chemical cross-linking with dimethyl suberimidate. The rate of cyclization of L-CA by the *C. fasciculata* enzyme was relatively high at acidic pH, decreasing to a very low rate at alkaline pH. In contrast, the rate of ring cleavage of L-DHO was very low at acidic pH and increased to a higher rate at alkaline pH. These pH–activity profiles gave an intersection at pH 6.6. The $K_m$ and $k_{cat}$ for L-CA were 0.846 ± 0.017 mM and 39.2 ± 6.4 $min^{-1}$, respectively; for L-DHO, they were 25.85 ± 2.67 μM and 258.6 ± 28.5 $min^{-1}$. The cryoprotectant dimethyl sulfoxide ($Me_2SO$), used as stabilizing agent in the complete purification and storage, markedly affected the DHOase activity. $Me_2SO$ increased the catalytic efficiency of the enzyme, as measured by $k_{cat}/K_m$, in the ring cyclization reaction but had no effect on the ring cleavage reaction. In spite of their marked physical differences, these kinetic and inhibitor studies with 5-substituted derivatives of orotic acid suggest that the protozoan, mammalian, and prokaryotic enzymes have a common catalytic mechanism.

Available treatments for human diseases caused by parasitic protozoa lag far behind the enormous advances made in combating bacterial infection. Trypanosomiasis and Leishmaniasis is treated, where possible, by compounds which have a high degree of mammlian toxicity. Also, malaria has once more become a global health problem due to the spread of organisms which are resistant to currently available antimalarial drugs. In general, drug-screening procedures have rarely been applied to these diseases, and there is a paucity of information on the biochemistry of these organisms.

Most parasitic protozoa are totally dependent on de novo biosynthesis for their pyrimidine requirements because they lack the relevant salvage enzymes, notably, thymidine kinase (Hill et al., 1981; Hammond & Gutteridge, 1982; Krungkrai et al., 1989). In mammals and other higher eukaryotes, the first three enzymes of pyrimidine biosynthesis, carbamoyl-phosphate synthetase II (CPS II, EC 6.3.5.5), aspartate transcarbamylase (ATCase, EC 2.1.3.2), and dihydroorotase [DHOase, 4,5-dihydroorotate amidohydrolase (EC 3.5.2.3)], are carried by a $M_r$ 240000 multifunctional protein (Kelly et al., 1986; Jones, 1980). There is little information as to the nature of these enzymes in parasitic protozoa. The fourth enzyme of the pathway, dihydroorotate dehydrogenase (EC 1.3.3.1), has been characterized in *Crithidia fasciculata* and *Trypanosoma brucei* (Pascal et al., 1983). This enzyme has been proposed as the site of action of a class of experimental antimalarial drugs, but there is little information on this protein in *Plasmodium* species. Orotate phosphoribosyltransferase (OPRTase, EC 2.4.2.10) and orotidine-5′-phosphate decarboxylase (ODCase, EC 4.1.1.23) catalyze the final steps of de novo synthesis of UMP in mammlian cells and exist as a bifunctional protein (Jones, 1980), but in *Plasmodium falciparum*, the most important cause of human malaria, these proteins have been described as discrete entities (Rathod & Reyes, 1983). With this limited information suggesting key differences between parasite and host in pyrimidine biosynthesis, and some evidence suggesting that parasitic protozoa might be acutely sensitive to existing or novel drugs which act at this site, we have focused our attention on this aspect of parasite metabolism. We now report the purification and characterization of a novel form of DHOase from two representative parasitic protozoa, *C. fasciculata* and *P. berghei*.

## Materials and Methods

*Materials.* Potassium [$^{14}$C]cyanate (56.7 Ci $mol^{-1}$), L-aspartate (220.4 Ci $mmol^{-1}$), [*carboxyl*-$^{14}$C]orotate (52.5 Ci $mol^{-1}$), and sodium [$^{14}$C]bicarbonate (55.5 Ci $mol^{-1}$) were purchased from Du Pont–New England Nuclear. DEAE-Sephacel was purchased from Pharmacia, and all other chromatographic operations were carried out using a fast protein liquid chromatography (FPLC) system on columns from Pharmacia. L-Carbamoylaspartate (L-CA), L-dihydroorotate (L-DHO), and orotate (L-OA) and its 5-substituted derivatives were purchased from Sigma. All chemicals, enzymes, and solvents were of analytical grade and were used without further purification. Double-distilled water was used throughout.

[*carboxyl*-$^{14}$C]Dihydroorotate (L-[$^{14}$C]DHO) was synthesized from [*carboxyl*-$^{14}$C]orotate as described (Smithers et al., 1978) and was purified by FPLC on a Mono Q 10/10 anion-exchange column with 0.2 M LiCl as eluent. The ra-

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diochemical purity of [*carboxyl*-$^{14}$C]dihydroorotate was >99%, as determined by thin-layer chromatography (TLC) on poly(ethylenimine)–cellulose (PEI) (Christopherson et al., 1978, 1981), and it had a specific radioactivity of 52.5 Ci mol$^{-1}$.

[$^{14}$C]Carbamoyl-L-aspartate (L-[$^{14}$C]CA) was synthesized from potassium [$^{14}$C]cyanate and L-aspartate as described (Christopherson et al., 1978). The radiochemical purity of L-[$^{14}$C]CA was 98.6%, as determined by TLC on PEI–cellulose, and it had specific radioactivity of 143.5 Ci mol$^{-1}$ as determined by using a colorimetric method (Prescott & Jones, 1969). L-[$^{14}$C]DHO and L-[$^{14}$C]CA were visualized on TLC using 5% (dimethylamino)benzaldehyde in 50% aqueous ethanol containing 1 M HCl.

*Enzyme Assays*: (*A*) *Carbamoyl-Phosphate Synthase.* Enzyme activity was measured with $NaH^{14}CO_3$ by following its conversion to [$^{14}$C]citrulline in the presence of L-ornithine and ornithine transcarbamylase (OTCase) (Mori et al., 1975). The standard assay contained 50 mM HEPES (pH 7.4), 2.5% glycerol, 7.5% $Me_2SO$, 2 mM DTT, 10 mM ATP, 15 mM $MgCl_2$, 4 mM $NaH^{14}CO_3$ (50 Ci mol$^{-1}$), 4 mM L-glutamine, 10 mM L-ornithine, and OTCase (2 units) in a total volume of 0.05 mL. Citrulline formation was measured after 15-min incubation at 37 °C.

(*B*) *Aspartate Transcarbamylase.* The assay was a modification of previously described methods (Mori et al., 1975; Christopherson et al., 1981). The reaction mixture contained 200 mM Tris-HCl (pH 8.5), 0.2 mM carbamoyl phosphate, 5 mM L-[$^{14}$C]aspartate, 2.5% glycerol, 7.5% $Me_2SO$, and enzyme to a total volume of 0.02 mL. The reaction mixture was incubated 15 min at 37 °C. The substrate, carbamoyl phosphate, and L-[$^{14}$C]aspartate were converted to L-[$^{14}$C]CA which was quantified by TLC using PEI–cellulose with 0.19 M LiCl/0.2% HCOOH as developing solvents.

(*C*) *Dihydroorotase.* DHOase activity was measured in the degradative (ring cleavage) direction as follows: The assay mixture contained 50 mM Tris-HCl (pH 9.0), 2.5% glycerol, 22.5% $Me_2SO$, 0.25 mM L-[$^{14}$C]DHO, and enzyme in a total volume of 0.02 mL. For DHOase activity in the forward direction, the assay mixture contained 50 mM MES–KOH (pH 6.0), 2.5% glycerol, 22.5% $Me_2SO$, 2.5 mM L-[$^{14}$C]CA, and enzyme in a total volume of 0.02 mL. The reaction mixtures were incubated at 37 °C for 15 min, and product formation was quantified by TLC.

(*D*) *Parasites. C. fasciculata* was cultivated and harvested as previously described (Pascal et al., 1983). *P. berghei* was cultivated in Balb/C mice. Cell-free extracts of parasites were prepared as described previously (Krungkrai et al., 1985) in the presence of the following protease inhibitors: 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.02 mg mL$^{-1}$ leupeptin, pepstatin, TLCK, and 1 mM EDTA; 10% glycerol and 30% $Me_2SO$ were routinely added to all preparations.

*Enzyme Purification*: *DHOase from C. fasciculata.* All operations were carried out at 4 °C. All buffer solutions contained 1 mM DTT and 1 mM EDTA. DHOase was purified from *C. fasciculata* to apparent homogeneity as follows:

(*A*) *Ammonium Sulfate Fractionation.* Freshly harvested cells (200 g) were suspended in 600 mL of 20 mM potassium phosphate (pH 7.0) containing 10 mM benzamidine, 0.02 g mL$^{-1}$ leupeptin, and 0.03 g mL$^{-1}$ PMSF and lysed by sonication (10 × 1 min pulses with 1-min cooling periods). After sonication, cellular debris was removed by centrifugation, and the supernatant was adjusted to 3% (w/v) with streptomycin sulfate and then to 30% saturation with $(NH_4)_2SO_4$. After being stirred for 45 min, the solution was centrifuged, and the resulting supernatant was adjusted to 60% saturation with $(NH_4)_2SO_4$. After being stirred for 1 h, the precipitated protein was pelleted by centrifugation, redissolved in the minimum quantity of 20 mM Bis-Tris/propane (pH 7.4), and dialyzed against this buffer (3 × 2 L).

(*B*) *DEAE-Sephacel.* The dialyzed protein solution was applied to a column (4 × 40 cm; flow rate 2 mL min$^{-1}$) of DEAE-Sephacel that had been equilibrated with 20 mM Bis-Tris/propane. After the column was washed with 1 L of buffer, bound proteins were eluted with a linear salt gradient (0–0.28 M KCl; 1.1 L in each chamber); 12-mL fractions were collected, and DHOase was found to elute at 0.18 M KCl. Active fractions were combined (260 mL), and the protein was precipitated by adjusting the solution to 85% saturating $(NH_4)_2SO_4$.

(*C*) *FPLC on Mono Q.* The precipitated enzyme was then dialyzed overnight against 50 mM Bis-Tris/propane (pH 7.4), 5% glycerol, and 30% $Me_2SO$ (3 × 2 L) applied to a Mono Q 10/10 FPLC column at a flow rate of 1.5 mL min$^{-1}$. The column was washed with 50 mM Bis-Tris/propane, pH 7.4, containing 5% glycerol and 30% $Me_2SO$ and eluted with a step gradient of 50 mM Bis-Tris/propane containing 0–0.6 M (50 mL), 0.6–1 M (12 mL), and 1 M (15 mL) KCl. Active fractions (which eluted at 0.3 M KCl) were pooled and applied to PD-10 desalting columns which had been equilibrated with 50 mM HEPES–KOH containing 1.5 M KCl and 5% glycerol, pH 7.4, to remove $Me_2SO$.

(*D*) *FPLC on Phenyl-Superose.* Active fractions from the previous step were chromatographed by FPLC on a phenyl-Superose 10/10 column which had been equilibrated with 50 mM HEPES–KOH containing 5% glycerol. Bound proteins were eluted by application of a linear gradient of 0–50% $Me_2SO$ and 1.5–0.25 M KCl in this buffer (flow rate 1 mL min$^{-1}$). Active fractions, which eluted at 25% $Me_2SO$ and 0.9 M KCl, were pooled, concentrated, desalted, and dialyzed with 25 mM Bis-Tris/propane, pH 6.5.

(*E*) *Chromatofocusing by FPLC on Mono P.* The enzyme solution was applied to a Mono P chromatofocusing column which had been equilibrated with 25 mM Bis-Tris/propane, pH 6.5. Bound proteins were eluted with 40 mL of Pharmacia polybuffer 74, pH 4.0, at a flow rate of 1 mL min$^{-1}$. Eluted fractions were neutralized immediately with 0.5 M HEPES–KOH containing $Me_2SO$, 50% v/v, pH 7.4. Active fractions, which eluted at p*I* 4.8, were pooled and concentrated on a Centricon 10 (Amicon).

(*F*) *Gel Filtration on Superose 12.* Final purification was effected by gel filtration on a Superose 12 column by elution with 50 mM HEPES–KOH containing 150 mM KCl, 5% glycerol, and 30% $Me_2SO$ at a flow rate of 0.5 mL min$^{-1}$. The column was calibrated by using the following proteins: thyroglobulin, 670 kDa; IgG, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; and vitamin $B_{12}$, 1350 Da. The enzyme eluted at position which corresponds to 42 000 Da (Figure 1).

*Separation of CPS II, ATCase, and DHOase Activities in P. berghei and Purification of DHOase.* Homogenates of host cell free parasites were chromatographed on a Superose 12 gel filtration column which had been equilibrated with 50 mM HEPES–KOH, pH 7.4, containing 10% glycerol and 30% $Me_2SO$. Proteins were eluted at a flow rate of 0.5 mL min$^{-1}$.

DHOase from *P. berghei* was purified 790-fold to near-homogeneity using the scheme which was developed for the *C. fasciculata* enzyme.

*Cross-Linking Studies and Other Methods.* Cross-linking conditions were adapted from Lee et al. (1985). The reaction was initiated by addition of dimethyl suberimidate to the

Table I: First Three Enzyme Activities of Pyrimidine Biosynthesis in *P. berghei* and Host Red Cells

| enzyme | *P. berghei* act. (nmol $min^{-1}$ $mg^{-1}$) | host red cell act. (pmol $min^{-1}$ $mg^{-1}$) |
|---|---|---|
| CPS II | 0.46 ± 0.15 (5)[a] | <1 (3) |
| ATCase | 7.24 ± 2.53 (6) | <5 (3) |
| DHOase | 0.44 ± 0.21 (5) | <1 (3) |

[a] Number of enzyme preparations in parentheses.

enzyme in Tris-HCl, pH 9.0, containing 30% $Me_2SO$. The mixture was incubated at room temperature for 3 h, and the reaction was then quenched by the addition of 1 M glycine. Cross-linking species were analyzed by SDS–PAGE and visualized by silver staining. Routine analytical electrophoresis was performed according to Laemmli (1970). Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as standard.

The activity of purified DHOase enzymes was tested in the range of pH 4.5–12.0 using equimolar mixtures of MES–KOH, HEPES–KOH, and Tris-HCl buffers (50 mM each). Velocity studies were performed at pH 6.0 (50 mM MES–KOH) and pH 9.0 (50 mM Tris-HCl) for the ring cyclization and ring cleavage reactions, respectively, in the absence of $Me_2SO$. Kinetic data from double-reciprocal and Dixon plots were fitted with linear regression analysis with $r > 0.98$.

## Results

*Separation and Stability of CPS II, ATCase, and DHOase Activities in P. berghei.* The apparent activities of the first three enzymes of pyrimidine biosynthesis, CPS II, ATCase, and DHOase, in the rodent malaria parasite *P. berghei* are shown in Table I. Initial characterization of these activities was hindered by their extreme lability. For this reason, we investigated the stability of the DHOase activity in another species of protozoa, *C. fasciculata*. Again the activity was found to be highly labile. At 4 °C, and in the presence of protease inhibitors, the *C. fasciculata* DHOase activity decreased 54% over 48 h. Following a survey of several known (and less well-known) stabilizing agents, dimethyl sulfoxide ($Me_2SO$) was found to preserve the DHOase activity; in 30% $Me_2SO$ and 5% glycerol, the DHOase activity was >90% retained after 60-days storage at 4 °C. For this reason, $Me_2SO$ was included in chromatographic solvents whenever possible. Having stabilized the activities, characterization of the first three enzymes was carried out by gel permeation chromatography (Figure 1). The three activities, CPS II, ATCase, and DHOase, eluted with relative molecular weights of 200K, 520K, and 40K, respectively (four to six determinations) which is consistent with the presence of three discrete protozoan enzymes. In control experiments with a freshly isolated mouse liver preparation, the three activities eluted at the same position on this system of apparent $M_r$ >670 K. This is consistent with previous reports (Jones, 1980) that these three activities are carried on a single multifunctional protein in mammalian systems.

*Purification of DHOase from P. berghei and C. fasciculata.* The small yield of *P. berghei* parasites from infected mice precluded the direct development of an efficient purification scheme for the DHOase of this organism. For this reason, the eventual purification scheme was developed in terms of the more easily obtained DHOase of *C. fasciculata* as this organism can be grown in large-scale (>100-g packed cell volume) culture.

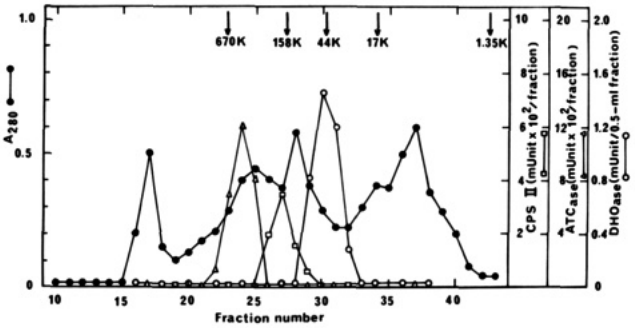


FIGURE 1: Profile of CPS II, ATCase, and DHOase activities of *P. berghei*, eluted from a Superose 12 column. The crude extract containing 12 mg of protein was applied to a Superose 12 column previously equilibrated with 50 mM HEPES–KOH (pH 7.4), 30% $Me_2SO$, 5% glycerol, and 150 mM KCl. The elution rate was 0.5 mL $min^{-1}$, and 0.5-mL fractions were collected. (●) $A_{280}$; (△) ATCase; (□) CPS II; (○) DHOase. The arrows indicate the positions of elution of marker proteins: thyroglobulin (670 kDa); IgG (158 kDa); ovalbumin (44 kDa); myoglobin (17 kDa); vitamin $B_{12}$ (1350 Da).

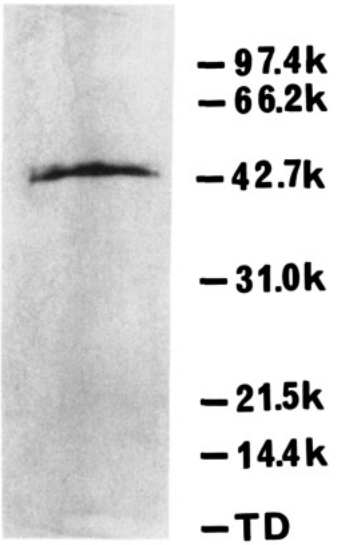


FIGURE 2: SDS–polyacrylamide gel electrophoretic analysis of purified *C. fasciculata* DHOase. A 12% polyacrylamide gel was run in 0.1% SDS according to the method of Laemmli (1970). Marker proteins were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and TD (tracking dye). The purified protein (1.8 μg) was stained with silver.

Following ammonium sulfate fractionation, chromatography on DEAE-Sephacel, FPLC on anion exchange and phenyl-Superose, chromatofocusing, and finally gel permeation chromatography, the *C. fasciculata* DHOase was purified ca. 16000-fold to apparent homogeneity (Table II). The purified

Table II: Purification of DHOase from *C. fasciculata*

| step | total protein (mg) | total act. (milliunits) | sp act. (milliunits/mg) | recovery (%) | *x*-fold purified |
|---|---|---|---|---|---|
| ammonium sulfate (30–60%) | 3877.0 | 1756 | 0.45 | 100 | 1 |
| DEAE-Sephacel | 560.0 | 1557 | 2.78 | 89 | 6 |
| Mono Q column | 73.0 | 1300 | 17.81 | 74 | 40 |
| phenyl-Superose column | 2.890 | 1139 | 394.12 | 65 | 880 |
| Mono P chromatofocusing | 0.384 | 664 | 1729.17 | 38 | 3800 |
| Superose 12 column | 0.067 | 494 | 7373.13 | 28 | 16000 |

Table III: Purification of DHOase from *P. berghei*

| step | total protein (mg) | total act. (milliunits) | sp act. (milliunits/mg) | yield (%) | x-fold purified |
|---|---|---|---|---|---|
| crude extract | 375.47 | 276.35 | 0.736 | 100 | 1 |
| Mono Q column | 90.44 | 213.62 | 2.362 | 77 | 3 |
| phenyl-Superose column | 6.75 | 177.04 | 26.228 | 64 | 36 |
| Mono P chromatofocusing | 1.30 | 143.15 | 110.114 | 52 | 150 |
| Superose 12 column | 0.21 | 121.20 | 577.143 | 44 | 784 |

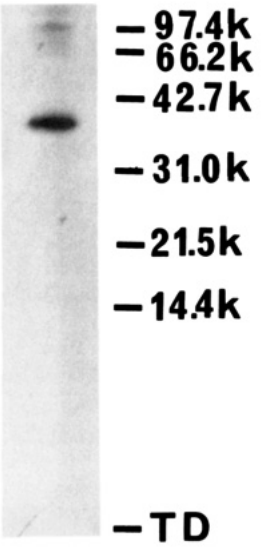


FIGURE 3: SDS–polyacrylamide gel electrophoresis of purified *P. berghei* DHOase. The bars indicate marker proteins on the 15% polyacrylamide gel, as shown in Figure 2. The protein (2.0 μg) was stained with silver.

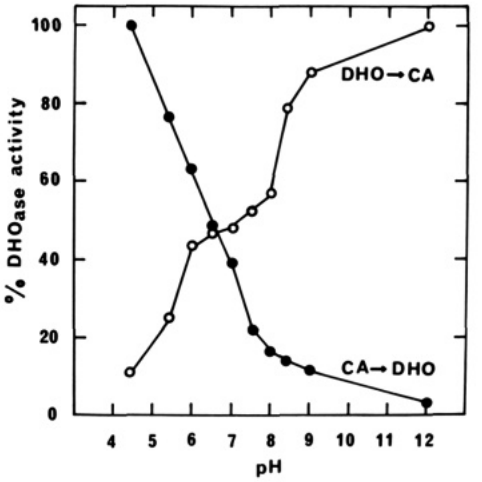


FIGURE 4: Effect of pH on the ring cyclization and cleavage activities of *C. fasciculata* DHOase. Incubation mixtures contained 50 mM buffer (MES–KOH for pH 4.4–6.0, HEPES–KOH for pH 6.0–8.0, and Tris-HCl for pH 8.0–12.0), 2.5 mM L-[$^{14}$C]CA or 0.25 mM L-[$^{14}$C]DHO, 22.5% $Me_2SO$, and 2.5% glycerol. The ring cyclization reaction profile is indicated by closed circles and the cleavage reaction profile by open circles. The maximal activities of the ring cyclization and cleavage reactions were 0.43 and 1.04 units (mg of protein)$^{-1}$, respectively, and were calculated to 100% enzyme activities.

enzyme appeared as a single band on SDS–PAGE when visualized by Coomassie blue or silver staining (Figure 2). Following optimization, this purification scheme was then applied to purify the DHOase from the malaria parasite. Even with these highly efficient methods, the limitation imposed by the availability of starting material necessitated the use of 450 *P. berghei* infected mice which, following complete exsanguination, gave sufficient material to allow purification and characterization of the *P. berghei* enzyme. *P. berghei* DHOase was purified ca. 790-fold to near-homogeneity (Table III). The purified enzyme appeared as a single band on SDS–PAGE when visualized by Coomassie blue; however, a faint impurity was detected by silver staining (Figure 3).

*Physical Characterization.* DHOases from *C. fasciculata* and *P. berghei* were found to have molecular weights of 44 000 ± 3000 ($n$ = 4) and 38 000 ± 3000 ($n$ = 3) by SDS–PAGE analysis. The native *C. fasciculata* enzyme was found to have a molecular mass of 42 000 ± 5000 daltons ($n$ = 4) as determined by gel permeation chromatography on a Superose 12 column. These results suggest that the trypanosomatid enzyme is active in monomeric form. Cross-linking studies confirmed this assessment. Using dimethyl suberimidate (0.05 mg mL$^{-1}$) incubated with the pure enzyme (0.028 mg mL$^{-1}$) for 3 h at room temperature (24 °C), we could not detected a cross-linked species following SDS–PAGE analysis and silver staining. The pure protein was determined to have p$I$ = 4.84 ± 0.29 ($n$ = 4), based on chromatofocusing studies, and was found to possess a blocked N-terminus. Similarly, the native molecular mass of the *P. berghei* enzyme was estimated to be 40 000 ± 4000 daltons ($n$ = 6). Thus, both protozoan DHOase enzymes appear to be active in monomeric form.

*Kinetic Analysis of C. fasciculata DHOase.* The activity of the *C. fasciculata* DHOase was measured over the pH range 4.5–12.0. In the ring cyclization reaction, very high activity appeared at acidic pH (4.0–6.0). In contrast, the rate of the ring cleavage reaction was very high at pH 9.0–12.0. The pH–activity profiles for both reactions intersected at pH 6.6 (Figure 4). The enzyme activity was strongly inhibited by preincubation with diethyl pyrocarbonate (DEPC); the activity was restored when the enzyme was preincubated with 2.5 mM DEPC and 10 mM L-CA (Table IV).

Table IV: Effect of Metal Chelators, Diethyl Pyrocarbonate (DEPC), and $Zn^{2+}$ on DHOase Activity

| compound | concn (mM) | % act.[a] |
|---|---|---|
| none | 0 | 100 |
| cysteine | 2.5 | 94 |
| EDTA | 2.5 | 89 |
| 1,10-phenanthroline | 2.5 | 92 |
| DEPC | 2.5 | 4 |
| DEPC and 10 mM L-CA[b] | 2.5 | 92 |
| $Zn^{2+}$ | 0.25 | 146 |
| | 0.50 | 225 |
| | 0.75 | 92 |
| | 1.0 | 65 |
| | 2.5 | 41 |

[a] DHOase assays, containing 2.5 mM [$^{14}$C]CA, 50 mM MES–KOH (pH 6.0), 22.5% $Me_2SO$, and the various compounds indicated, were performed at 37 °C for 15 min. The results were compared to the enzyme activity in the absence of tested compounds. [b] The enzyme was preincubated with 2.5 mM DEPC in the absence or presence of 10 mM L-CA at 4 °C for 10 min and then assayed with 2.5 mM [$^{14}$C]CA for 15 min at 37 °C.

At pH 6.0, the enzyme displayed normal Michaelis–Menten saturation kinetics for the cyclization of L-CA to DHO. From Lineweaver–Burk plots, the enzyme was estimated to have an apparent $K_m$ = 0.846 ± 0.017 mM and $k_{cat}$ = 39.2 ± 6.4 min$^{-1}$. For the reverse reaction (L-DHO → L-CA) at pH 9.0, the enzyme had apparent $K_m$ and $k_{cat}$ values of 25.85 ± 2.67 μM

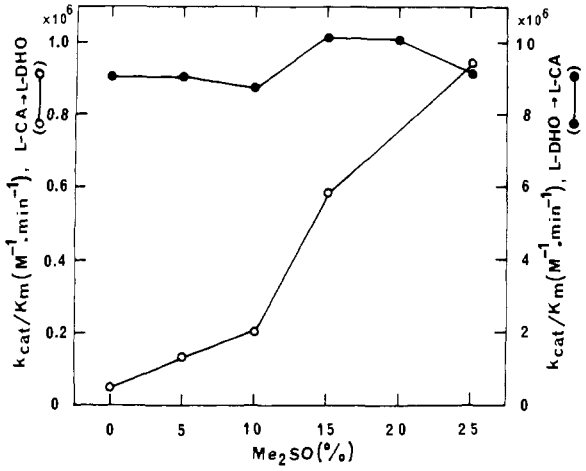


FIGURE 5: Catalytic efficiency, measured by $k_{cat}/K_m$, of *C. fasciculata* DHOase activity (both ring cyclization and cleavage reactions) in various concentrations of $Me_2SO$. The ring cyclization reaction (L-CA → L-DHO) (○) and ring cleavage reaction (L-DHO → L-OA) (●) were measured at pH 6.0 and pH 9.0, respectively.

and 258.6 ± 28.5 $min^{-1}$, respectively.

The metal chelators cysteine, EDTA, and 1,10-phenanthroline showed no effect on the enzymatic activity (tested at 2.5 mM). However, the enzyme was activated by low concentrations of $Zn^{2+}$. At higher concentrations (>0.5 mM), this ion inhibited both forward and reverse reactions (Table IV). The inhibition was competitive in nature with an apparent $K_i$ = 3.25 mM.

The stability conferred on both protozoan DHOase enzymes by $Me_2SO$ prompted us to analyze the effect of this reagent on the kinetic parameters of the *C. fasciculata* enzyme. The effects of varying the $Me_2SO$ concentration on the ring cyclization and ring cleavage reactions were found as follows. With increasing [$Me_2SO$] (0 → 25%), the $K_m$ for L-CA was reduced from 0.83 to 0.26 mM, and the maximum initial velocity ($V_{max}$) increased from 0.9 to 5.6 μmol $min^{-1}$ $mg^{-1}$. In the reverse reaction, increasing [$Me_2SO$] (0 → 25%) led to reduction of both the apparent $K_m$ for L-DHO (from 28 to 13 μM) and also the $k_{cat}$ (from 260 to 120 $min^{-1}$). Thus, in this case, $Me_2SO$ acts as an uncompetitive inhibitor. In summary, $Me_2SO$ increases the catalytic efficiency of the enzyme, as measured by $k_{cat}/K_m$, in the ring cyclization reaction but has no effect on the ring cleavage reaction (Figure 5).

*Kinetic Analysis of P. berghei DHOase.* Due to the extreme lability of the *P. berghei* DHOase, it was necessary to carry out all kinetic experiments in the presence of 22.5% $Me_2SO$. The pH–activity profile of the *P. berghei* DHOase was similar to that obtained from analysis of the *C. fasciculata* enzyme. Rates of ring cyclization (at pH 6.0) and ring cleavage (at pH 9.0) were measured using L-CA and DHO, respectively. Under these conditions, $k_{cat}$ values for forward and reverse reactions were determined to be 244 and 286 $min^{-1}$, respectively. To compare inhibitor studies with the mammalian system (Christopherson et al., 1989), the effect of orotic acid and various 5-substituted orotate derivatives on the forward and reverse reactions was investigated with *P. berghei* DHOase. All of these compounds were found to be competitive inhibitors of the forward and reverse reactions. Apparent $K_i$ values were obtained from Dixon plots (Table V).

Table V: Kinetic Constants of L-CA, L-DHO, and OA and Its 5-Substituted Derivatives in Ring Cyclization (pH 6.0) and Cleavage (pH 9.0) Reactions Catalyzed by *P. berghei* DHOase in the Presence of 22.5% $Me_2SO$

| | ring cyclization | | ring cleavage | |
|---|---|---|---|---|
| compound | $K_m$ (mM) | $K_i$ (mM) | $K_m$ (mM) | $K_i$ (mM) |
| L-CA | 0.293 | | | |
| L-DHO | | 0.060[a] | 0.014 | |
| OA | | 0.525 | | 1.400 |
| 5-F-OA | | 0.103 | | 0.060 |
| 5-$NH_2$-OA | | 0.355 | | 0.118 |
| 5-$CH_3$-OA | | 0.975 | | 0.122 |
| 5-Br-OA | | 0.750 | | 1.675 |
| 5-I-OA | | >3.500 | | 2.450 |

[a] L-DHO is a strong competitive inhibitor on the ring cyclization reaction from L-CA → L-DHO at pH 6.0, whereas L-CA has no effect on the ring cleavage reaction at pH 9.0.

## Discussion

Recent studies have highlighted the potential vulnerability of parasitic protozoa toward agents which affect pyrimidine biosynthesis. Compounds which are presumed to affect either DHOase or DHO dehydrogenase activity have been shown to be selectively toxic toward plasmodium species causing human malaria (Rathod et al., 1989; Gutteridge, 1989). In order to fully exploit this pharmacological target, detailed mechanistic and structural characterization of the plasmodium enzymes responsible for pyrimidine biosynthesis is required. However, such an undertaking has been considered prohibitive because of the extreme scarcity and lability of these parasite enzymes. For this reason, we invested effort developing optimal solvent systems (aqueous dimethyl sulfoxide) and purification schemes which facilitated the characterization of the process of pyrimidine biosynthesis in plasmodium. With these methods and solvent systems, the first three enzymes of pyrimidine biosynthesis in the rodent malaria parasite *P. berghei* (CPS II, ATCase, and DHOase) could be readily separated in highly active form by gel permeation chromatography (Figure 1). A similar result was obtained on analysis of these activities in the insect trypanosomatid *C. fasciculata*. Both of these organisms were chosen as initial biochemical sources because of their relative ease of culture and because both have proved reliable models of the human pathogens in other studies of parasite biochemistry.

It appears that de novo biosynthesis of the pyrimidine ring is catalyzed in parasitic protozoa by six discrete enzyme activities. This is similar to what is found in many prokaryotic systems but differs from the analogous mammalian process in which the first three activities are associated with a single multifunctional protein (Jones, 1980). Following purification, the protozoan DHOase was found to have several features in common with previously reported prokaryote counterparts, but notable differences were also evident. Physically, the *C. fasciculata* enzyme was found to be active in monomeric form with molecular mass 44 000 Da. Similarly, the plasmodium enzyme is an active monomer of 38 000 Da. Furthermore, in the course of purification of *P. berghei* dihydroorotate dehydrogenase (DHODase) using 2.5 mM L-DHO added into all buffers to stabilize the DHODase, we found that the DHOase activity was repeatedly eluted at a molecular weight of 40 000 on a gel filtration FPLC column, confirming that the protozoa enzyme is the active monomer (Krungkrai et al., unpublished results). All previously described prokaryote enzymes are active in dimeric form. The protozoan DHOase catalyzes the reversible reaction L-CA ⇌ L-DHO. The pH–activity profiles had intersects at a pH of 6.6, and the enzyme was readily deactivated by diethyl pyrocarbonate (DEPC). These results are suggestive of the involvement of an active-site histidine residue. Notwithstanding the marked physical differences, these properties and the kinetic parameters of the protozoan DHOase suggest that the protozoan, mammalian, and prokaryotic DHoase enzymes share a common catalytic

mechanism [this was also reflected in the inhibitory effect of various 5-substituted derivatives of orotic acid on the plasmodium DHOase (Table V) and mammalian DHOase (Christopherson et al., 1989)]. The catalytic process may involve the action of an active-site $Zn^{2+}$ ion which can coordinate to a putative tetrahedral intermediate formed, following (in the forward direction) attack by the ureido amino function at the $\beta$-carboxyl group of L-CA or following (in the reverse direction) attack by water at this carboxyl function (Walsh, 1979).

In the present investigation, the finding that dimethyl sulfoxide conferred exceptional stability on the protozoan DHOase prompted us to examine the kinetic properties of the enzyme in the presence of this cosolvent. Dimethyl sulfoxide reduced the $K_m$ for both substrates. The organic cosolvent also caused an increased $k_{cat}$ for the forward (water elimination) reaction and a decreased $k_{cat}$ in the reverse (hydrolysis) direction. Plots of $k_{cat}/K_m$ vs cosolvent concentration are linear (Figure 5), suggesting that the changes in catalytic parameters are not due to a hydrophobic partitioning effect on substrate binding but to a direct effect on the solvation state of the active-site region of the enzyme. On the basis of the postulated catalytic mechanism, the cosolvent effects may therefore be interpreted as follows: If the active-site region of the protein is highly solvated, then the effect on $K_m$ may arise because the organic cosolvent displaced water bound in the active site, thereby facilitating entry and/or binding of substrate. Similarly, the effect on $K_{cat}$ would reflect the relative ease of entry or exit of water in the substrate-bound active-site region of the protein. Dimethyl sulfoxide has been noted to have similar effects on catalysis in previous cryoenzymology studies (Fink & Geeves, 1983).

The physical and chemical properties of DHOase enzymes resemble those of metalloproteases such as carboxypeptidases A and B, angiotensin-converting enzyme, thermolysin, and enkephalinase (Christopherson et al., 1989). Potent inhibitors of these zinc-containing proteases have been developed, some of which have important clinical use. These compounds were developed on a semirational basis with the aid of detailed structural information on the target proteins. By extrapolation from these studies, some fairly potent inhibitors of mammalian DHOase have also been described (Christopherson et al., 1989). The physical characteristics of the protozoan DHOase (relatively small monomeric protein) give reason to believe that this enzyme, in contrast to the mammalian counterpart, might be a suitable and pharmacologically important candidate for structural analysis. This will require amino acid sequencing, gene cloning, and expression of the protein in a suitable vector. We are currently pursuing this objective.

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