Purification and characterization of dihydroorotase from Clostridium oroticum, a zinc-containing metalloenzyme

William Edward Balch
Portland State University

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Dihydroorotase (4,5-L-dihydro-orotate amidohydrolase, EC 3.5.2.3) which catalyzes the reversible cyclization of N-carbamyl-L-aspartate to L-dihydro-orotate has been purified from orotate-grown Clostridium oroticum by a combination of streptomycin sulfate fractionation, DEAE-Sephadex chromatography, and hydroxylapatite chromatography. The enzyme has been shown to be homogeneous when subjected to polyacrylamide gel electrophoresis. Thin-layer gel chromatography with Sephadex G-200 indicated the enzyme to have a molecular weight of 110,000 ± 10,000. Sodium
Dodecyl sulfate gel electrophoresis using two different buffer systems indicate the enzyme to be composed of two identical subunits with a molecular weight of 56,000 ± 5300. Dihydroorotase has been shown to be a zinc-containing metalloenzyme by atomic absorption spectroscopy with two g atoms of zinc per 56,000 g of protein. The pH optima for the conversion of N-carbamyl-L-aspartate to L-dihydroorotate and L-dihydroorotate to N-carbamyl-L-aspartate have been determined to be at pH 6.0 and pH 8.2 respectively. The binding constant of the enzyme for each substrate has been investigated with values of 0.13 mM for N-carbamyl-L-aspartate and 0.07 mM for L-dihydroorotate.
PURIFICATION AND CHARACTERIZATION
OF DIHYDROOROTASE FROM CLOSTRIDIUM OROTICUM,
A ZINC-CONTAINING METALLOENZYME

by
WILLIAM EDWARD BALCH

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOL0GY

Portland State University
1973
TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the thesis of William Edward Balch presented 4 June 1973.

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Earl Fisher, Jr.

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Earl Fisher, Jr., Head, Department of Biology

David T. Clark, Dean of Graduate Studies
ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the excellent assistance and time of Dr. J. Loehr for determination and interpretation of the atomic absorption spectra. I would also like to thank Dr. H. Mason at the University of Oregon Medical School for the use of his Atomic Absorption Spectrophotometer for the determination of zinc content of dihydroorotase.
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INTRODUCTION

Dihydroorotase (4,5-dihydroorotate amidolyase, EC 3.5.2.3) catalyzes the reversible cyclization of N-carbamyl-L-aspartic acid to L-dihydroorotic acid.

\[
\begin{align*}
\text{N-carbamyl-aspartic acid} & \rightleftharpoons \text{dihydroorotic acid} \\
\end{align*}
\]

The reaction is an intermediate step in the biosynthesis and degradation of pyrimidines as outlined in Figures 1 and 2. Early evidence from a

\[
\begin{align*}
\text{Aspartate} + \text{Carbamyl-phosphate} \rightarrow \text{N-carbamyl-aspartate} \\
\text{N-carbamyl-aspartate} \rightarrow \text{Dihydroorotate} \\
\text{Dihydroorotate} \rightarrow \text{Orotate} \\
\text{Orotate} \rightarrow \text{Uridine Triphosphate} \\
\text{Cytidine Triphosphate} \\
\end{align*}
\]

\[
\begin{align*}
\text{Orotate} + \text{NADH (or NADPH)} \rightarrow \text{Dihydroorotate} + \text{NAD}^+ (\text{or NADP}^+) \\
\text{Dihydroorotate} + \text{H}_2\text{O} \rightarrow \text{Orotate} \\
\text{N-carbamyl-aspartate} \rightarrow \text{Aspartate} + \text{CO}_2 + \text{NH}_3 \\
\end{align*}
\]

Fig. 1. Pathways for pyrimidine metabolism in bacteria:
(A) pyrimidine biosynthesis, (B) orotate degradation.
Fig. 2. The general scheme of pyrimidine metabolism in organisms.
number of laboratories indicated that orotate was a precursor of nucleic acid pyrimidines (1-6). The pathway for orotate catabolism was first demonstrated by Lieberman and Kornberg (7-9), who studied the orotate fermenting bacterium Clostridium oroticum (formerly Zymobacterium oroticum (10)) which was isolated from mud (11), and gave evidence for the functional role of N-carbamyl-aspartate and dihydroorotate in the pyrimidine pathway. Yates and Pardee (12) observed that two of the enzymes, dihydroorotase and dihydroorotic dehydrogenase, were produced in much higher levels in extracts of Clostridium oroticum when the organism was cultured on orotate as the sole carbon and energy source then when it was cultured glucose. Subsequently, they investigated the regulatory control of pyrimidine biosynthesis in E. coli B. Their work has been more recently expanded by Beckwith et al. (13) and Taylor et al. (14) where the role of repressor control of the enzymes in E. coli B has been firmly established. In addition to understanding the regulatory mechanisms involved in pyrimidine biosynthesis, a number of other aspects of the pathway have been worked out, including detailed analyses of the structural and catalytic properties of two of the enzymes, aspartate transcarbamylase and dihydroorotate dehydrogenase. Presently, pyrimidine biosynthesis through the orotate pathway (Fig. 2) has been demonstrated in a number of organisms to involve a set of constitutive enzymes which are present under all growth conditions, and thus involved in the biosynthesis of pyrimidines. Another set of enzymes are present only in a limited number of bacterial species when cultured on orotate. These enzymes are inducible and are primarily involved in the degradation of orotate.

Compared to other enzymes of the pyrimidine pathway, little has
been done on the dihydroorotase to determine its characteristics or role in the biosynthesis and degradation of pyrimidines. Dihydroorotase was first demonstrated to be involved in orotate degradation by Lieberman and Kornberg (7-9) using extracts of Cl. oroticum. Subsequently, the degradative enzyme was partially purified by Sander et al. (15) from extracts prepared from Cl. oroticum cultured on orotate. Their work indicated a divalent metal ion requirement, probably Zn$^{2+}$, for enzyme activity.

The biosynthetic dihydroorotase from E. coli B has been purified by Sander and Heeb (16), and by Mazus and Buchowicz (17, 18) from pea plants. Activity of the biosynthetic enzyme from pea seedlings or from E. coli B was neither stimulated by addition of Zn$^{2+}$ nor inhibited by the addition of metal ion chelators. Biosynthetic dihydroorotase has also been demonstrated in extracts of rat liver cells (19, 20), erythrocytes and leukocytes (21-24), and Novikoff ascites tumor cells (25, 26).

In the present study the degradative dihydroorotase from Cl. oroticum, cultured with orotate as a sole source of carbon and energy, has been purified to homogeneity. The purified enzyme has been analyzed for metal ions necessary for enzyme activity, molecular weight, subunit composition, and kinetic constants. Since Cl. oroticum is an organism in which dihydroorotase has been demonstrated and which can metabolize orotate, the degradative enzyme has been purified and partially characterized in order to compare it to the constitutive biosynthetic dihydroorotase enzymes from E. coli B and pea plants, and to other enzymes of the degradative pyrimidine pathway in Cl. oroticum.
MATERIALS AND METHODS

Culture Methods

*Clostridium oroticum* was cultured at 30° in 10 liter and 40 liter carboys using the following media: tryptone, 50 g; yeast extract, 5 g; Na-thioglycollate, 5 g; KH₂PO₄, 13.6 g; K₂HPO₄ (anhydrous), 69.5 g; Na-orotate, 51.3 g; H₂O, 10 liters. Final pH of the media is 7.5. *C. oroticum* was inoculated into freshly autoclaved media, and maintained under intermittent stirring until exhaustion of Na-orotate as indicated by the cessation of gas production. Subsequently, 13 g/10 liters Na-orotate was added and cells were harvested when vigorous gassing resumed, and after most of the added Na-orotate had disappeared. Cells were harvested using a CEPA continuous flow centrifuge (Carl Padberg GMBH, 7630 Lahr/Schwarzwald) with type K head at 40,000 rpm. Flow rate was maintained at 400 ml/minute. The pellet was washed twice with 0.05 M sodium phosphate buffer (pH 7.0) and stored at -20° until use. Cell yield was approximately 23 g wet weight/10 liters media.

Preparation of Cell-Free Extracts

Washed cells which had been stored at -20° were suspended to approximately 0.3 g wet cells per ml in 0.05 M sodium phosphate buffer (pH 7.0) and disrupted with a French press (American Instrument Co., Inc.) at 7000 psi of pressure. Crude extract was centrifuged for 30 minutes at 30,000 x g followed by ultracentrifugation for 90 minutes at 150,000 x g. Pellets were discarded. The supernatant fluid was stored at -20° and used as the starting material for enzyme purification.
Purification of Dihydroorotase

The 150,000 x g supernatant was stirred in an ice bath while 0.25 volumes of a 20% streptomycin sulfate solution was slowly added. Mixing was continued for 15 minutes after the final addition of streptomycin sulfate. The solution was then allowed to set for 45 minutes in an ice bath. The precipitated nucleic acids were removed by centrifugation for 20 minutes at 30,000 x g. The pellet was discarded and the supernatant transferred to a Sephadex G-25 column equilibrated at room temperature with 0.025 M Tris-HCl buffer (pH 7.8) for removal of streptomycin sulfate. The enzymatically active fractions were combined.

Combined fractions were made 0.15 M NaCl and 0.5 mM Zn$^{2+}$, and layered on Sephadex DEAE A-50 equilibrated at room temperature with 0.025 M Tris-HCl buffer (pH 7.8) which was 0.15 M NaCl and 0.5 mM Zn$^{2+}$. The column was washed with the equilibration buffer listed above followed by a linear salt gradient from 0.15 M NaCl to 0.5 M NaCl in 0.025 M Tris-HCl buffer (pH 7.8) and 0.5 mM Zn$^{2+}$. Slope of the gradient was 0.0014 M increase in NaCl concentration per ml with a flow rate of 30 ml per hour. Dihydroorotase remained bound to the column during this procedure. Enzyme was eluted off DEAE by removing the gel from the column, adding NaCl to a final concentration of 1.0 M, and rapidly washing the gel on a Buchner funnel with a minimum volume (50 to 75 ml) of 1.0 M NaCl in 0.025 M Tris-HCl buffer (pH 7.8). Under these conditions the enzyme was found to be stable for at least 2 weeks at room temperature.

Protein recovered from DEAE was dialyzed 24 hours against 0.01 M potassium phosphate buffer (pH 6.8) at 4°, the temperature of all subsequent purification procedures. A precipitate which occurred upon
dialysis was removed by centrifugation at 30,000 x g for 20 minutes and discarded.

Supernatant from the previous step was transferred to a hydroxyapatite column equilibrated with 0.01 M potassium phosphate buffer (pH 6.8). Approximately 15 to 20 cm hydroxyapatite in a KL5/30 Pharmacia column supported on a 1 cm layer of Sephadex G-25 was found to accommodate 50-100 mg of protein. Proteins were eluted using a 0.01 M to 0.5 M potassium phosphate buffer (pH 6.8) gradient with a linear increase of 0.002 M potassium phosphate per ml. Flow rate was adjusted to 16 to 20 ml per hour. Elution pattern was followed by conductance readings using a conductivity bridge (Model 31, Yellow Springs Instrument Co., Inc.). Active fractions were combined and made 50 µM Zn²⁺. After overnight storage at 4° in this buffer activity was consistently observed to increase two to three fold.

Combined fractions from the first hydroxyapatite column were dialyzed against 0.01 M potassium phosphate buffer (pH 7.8) and transferred to a second hydroxyapatite column (KL5/30) equilibrated with 0.01 M potassium phosphate buffer (pH 7.8). Protein was eluted using a 0.01 M to 0.25 M potassium phosphate buffer (pH 7.8) gradient. Purified enzyme was made 50 µM Zn²⁺ and stored at 4°. Activity was stable for at least 4 weeks when stored in 0.15 M to 0.2 M potassium phosphate buffer (pH 6.8 or 7.8) and 50 µM Zn²⁺.

**Enzyme Assays**

Dihydroorotase activity was assayed in both the biosynthetic and degradative directions using similar preincubation and reaction conditions, but different means of detecting the product. The biosynthetic conversion
of N-carbamyl-L-aspartate to L-dihydroorotate was measured using a modification of the direct assay as outlined by Sander et al. (15). Increase in absorbance at 230 nm due to the formation of L-dihydroorotate was followed for 3 minutes using a Coleman-Hitachi 124 Spectrophotometer. The reported extinction coefficient for L-dihydroorotate is 1.17 mM⁻¹cm⁻¹ at 230 nm, and the absorbance response was found to be linear from 0 to 54 mM dihydroorotate (15). The reaction mixture contained 67 µmoles of MES(2(N-morpholino) ethane sulfonic acid)-NaOH buffer (pH 5.8-6.0), 20 µmoles of N-carbamyl-D,L-aspartate, and enzyme. The reaction was initiated by the addition of substrate to the mixture. Normally, a 3 minute incubation at room temperature of enzyme in buffer preceded initiation.

Assay of degradative activity, the conversion of L-dihydroorotic acid to N-carbamyl-L-aspartate, used essentially the same reaction conditions as in the direct assay. Detection of the product (N-carbamyl-L-aspartate) was carried out using the procedure of Prescott and Jones (27), a very sensitive assay for ureido and carbamyl compounds. Enzyme in 67 mM MES-NaOH buffer (pH 5.8-6.0), except where indicated otherwise, was initiated by the addition of 24 µmoles L-dihydroorotate to a total volume of 5 ml after 3 minutes preincubation at room temperature. One ml aliquotes were removed at 30 second intervals for 2 minutes and pipetted into one ml of the antipyrine-oxime reagent which terminated the reaction. The mixture of 2 parts antipyrine (1,5-dimethyl-2-phenyl-3-pyrazolone) to 1 part oxime (diacetyl-monoxime) was made just prior to use in an ice bath and in the dark. Subsequently, all procedures involving the use of the reagent were carried out in a dimly lit room. The more sensitive assay
(method II) was used at all times and standard curves were included during each assay. After a 15 hour incubation in the dark at room temperature followed by 75 minutes in a $45^\circ$ water bath, under even illumination, absorbance was read at 466 nm using a Gilford 2000 Spectrophotometer equipped with digital readout.

A unit of dihydroorotase activity is reported as 1 nmole of L-dihydroorotate formed per minute under the conditions given for the direct assay.

**Protein Determination**

Elution profiles were followed at 260 and 280 nm according to the method of Warburg and Christian (28), and at 220 nm using as a standard bovine serum albumin. Protein concentration for determination of specific activity was measured by use of the Folin phenol reagent as described by Lowry et al. (29) with bovine serum albumin as the standard.

**Acrylamide Gel Electrophoresis**

Analytical disc electrophoresis was carried out with a refrigerated Polyanalyst apparatus (Buchler Instruments) by using the general procedures of Davis (30). The upper and lower gel composition, and the Tris buffer system were as described by Jovin et al. (31). Gels were stained and destained by the procedure of Chrambach et al. (32), and stored in 7.5% acetic acid. A Densicord Model 542 (Photovolt Corporation) was used to trace the gels. Enzyme activity was detected by slicing the gel into 2 mm segments using a gel-slicer fabricated at the Portland State University Science Shop, and assaying for activity according to the Prescott and Jones procedure (27) with L-dihydroorotate as the substrate.
Both protein homogeneity and enzyme activity were followed in 7.5% and 12% gels.

**Subunit Determination**

Subunit analysis was carried out using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Composition and molecular weight of the subunit structure of dihydroorotase was determined by both the procedure of Shapiro et al. (33) as outlined by Weber and Osborn (34), and the procedure of Neville (35) using a discontinuous buffer system. Conditions as stated in the papers were followed explicitly. Gels are described by the notation of Hjerten (36), in which the first numeral (T) denotes the total weight of monomer (acrylamide plus N, N'-methylenebisacrylamide) per 100 ml of solvent, and the second numeral (C) denotes the amount of N, N'-methylenebisacrylamide expressed as a percentage (w/w) of the total weight of the monomer. Gel composition using the Neville procedure was 11.1 x 0.9. Gel composition using the Weber and Osborn procedure was 11.4 x 0.25. Staining and destaining of the gels was done according to Weber and Osborn, except the concentration of Coomassie Blue R was reduced to 0.01% while staining time was increased to 12 hours. Destaining was carried out for 24-48 hours using a diffusion destainer (Hoefer Scientific Instruments, Inc.).

**Molecular Weight Determination**

The molecular weight of dihydroorotase was determined by thin-layer gel filtration using Pharmacia thin-layer gel apparatus and following the procedure as outlined by Pharmacia (37). Sephadex G-200 superfine (10-40µ bead size) was spread in a 0.6 mm layer on a 20 cm plate and equilibrated
for 2 days with 0.2 M potassium phosphate buffer (pH 6.8) which contained 50 µM Zn\(^{2+}\). After equilibration, protein standards (5 mg/ml) and purified enzyme were placed carefully on the plate using a fine tipped pasteur pipette. The plate angle was adjusted to 20°, producing a flow rate of 3.0 cm per hour for the excluded thyroglobulin. Enzyme was detected by removing 0.5 x 2 cm sections of the gel, transferring it to the standard reaction mixture with L-dihydroorotate as the substrate, and detecting the formation of N-carbamyl-L-aspartate according to the procedure of Prescott and Jones (27). Sephadex gel under these conditions was found not to interfere with the assay. The peak of activity was used as the center of the protein spot. Molecular weight markers were detected by absorbing them onto filter paper (Whatman 3 MM) and staining with 0.25% Coomassie Brilliant Blue R in methanol:acetic acid (90:10, v/v) for 15 minutes. The paper was destained by a 5 minute wash in cold tap water followed by a 12 hour soak in ethanol:acetic acid:H\(_2\)O (50:10:50, v/v/v). Inverse migration distance (relative to thyroglobulin) was plotted against log MW.

**Atomic Absorption Spectroscopy**

Purified dihydroorotase was concentrated to approximately 0.75 mg/ml using an Amicon ultrafiltration apparatus (Amicon Corp.) with a PM-10 membrane. The storage buffer (0.2 M potassium phosphate buffer (pH 6.8) containing 50 µM Zn\(^{2+}\)) was exchanged for 0.025 M Tris-HCl buffer (pH 7.8) on a Sephadex G-25 column. Fractions from the column were measured for the presence of zinc at 2138.6 Å using a Varian-Techton Atomic Absorption Spectrophotometer (Type AA-5, Cary Instruments, Inc.).
A standard curve for zinc using a Techton ZnNO$_3$ standard (15.3 mM) was run in the presence of eluting buffer. Samples were run a minimum of 3 times and data was averaged. No interferences with the zinc determination were detected using the method of additions as described by Willis (38).

Materials

All chemicals were obtained commercially, and unless otherwise stated they were either analytical or reagent grade. The following were obtained from Sigma Chemical Co.: streptomycin sulfate, Sephadex DEAE A-50, Sephadex G-25-300, Sephadex G-200-40, Trizma (Tris(hydroxymethyl)-aminomethane) base, MES (2(N-Morpholino) ethane sulfonic acid), N-carbamyl-D,L-aspartate, L-dihydroorotate, Coomassie Brilliant Blue R, 2-mercaptoethanol (2-hydroxyethylmercaptan), and sodium dodecyl sulfate (lauryl sulfate). Hydroxylapatite was purchased from Bio-Rad. Acrylamide and N,N'-methylenebisacrylamide were obtained from Eastman Organic Chemicals and were recrystallized from chloroform and acetone respectively as described by Loening (39). TMEDA (N,N,N',N'-tetramethylethylenediamine) was Bio-Rad electrophoresis grade. Antipyrine (1,5 dimethyl-2-phenyl-3-pyrazolone), diacetylmonoxime, and bromphenol blue were products of Matheson Coleman & Bell. ZnSO$_4$·7H$_2$O was obtained from Baker & Adamson. Protein standards were purchased as follows: chymotrypsinogen, bovine serum albumin, ovalbumin, pepsin, ribonuclease A, and thyroglobulin, Sigma; trypsin, Worthington; aldolase and blue dextran, Pharmacia calibration kit.
RESULTS

Purification of dihydroorotase

The results of a typical preparation of dihydroorotase from *Cl. oroticum* are shown in Table I. A 102-fold increase in specific activity over that measurable in crude extract with a recovery of 21% was observed with the most purified fraction. Storage of the 150,000 x g extract at -20° resulted in progressive enzyme decay. However, addition of streptomycin sulfate to remove nucleic acids resulted in complete recovery of the original amount of enzyme present prior to storage. The degree of stimulation was observed to be directly proportional to the amount of enzyme decay. In some cases stimulation was on the order of three to four fold to bring activity back to the original level. Chromatography on DEAE-Sephadex of the G-25 fraction in 0.5 mM Zn²⁺ resulted in very strong binding of dihydroorotase to the ion exchanger. A gradient up to 0.5 M NaCl would not elute the protein from the DEAE-Sephadex, whereas in the absence of Zn²⁺ dihydroorotase was found to elute in a single peak between 0.25 M NaCl and 0.35 M NaCl. Normal recovery from DEAE-Sephadex by washing with 1.0 M NaCl gave 100% to 150% recovery of the enzyme units, which was lowered to 60-70% after dialysis against 0.01 M potassium phosphate (pH 6.8). The elution patterns of hydroxylapatite columns at pH 6.8 and pH 7.8 are given in Figures 3 and 4, respectively. Since hydroxylapatite has a high affinity for divalent metal ions such as Ca²⁺ and Zn²⁺, the specific activity was consistently observed to increase two to three fold after overnight incubation of the recovered fractions in the presence of 50 µM Zn²⁺. Peak B on the second hydroxylapatite represents purified enzyme. Stability of the purified
TABLE I

PURIFICATION OF DIHYDROOROTASE FROM CLOSTRIDIUM OROTICUM

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Fraction</th>
<th>Total units*</th>
<th>Total protein (mg)</th>
<th>Specific activity</th>
<th>% recovery</th>
</tr>
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<tr>
<td>1.</td>
<td>Supernatant fluid (150,000 x g)</td>
<td>12,100</td>
<td>740</td>
<td>16.4</td>
<td>100</td>
</tr>
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<td>2.</td>
<td>Streptomycin sulfate supernatant</td>
<td>12,500</td>
<td>740</td>
<td>16.9</td>
<td>107</td>
</tr>
<tr>
<td>3.</td>
<td>Sephadex G-25 eluant</td>
<td>9,000</td>
<td>490</td>
<td>18.4</td>
<td>75</td>
</tr>
<tr>
<td>4.</td>
<td>DEAE Sephadex eluant</td>
<td>18,000</td>
<td>58</td>
<td>310</td>
<td>148</td>
</tr>
<tr>
<td>5.</td>
<td>0.01 M potassium phosphate buffer (pH 6.8) dialysate</td>
<td>7,800</td>
<td>48.5</td>
<td>161</td>
<td>65</td>
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<td>6.</td>
<td>First hydroxylapatite eluant (pH 6.8)</td>
<td>3,600</td>
<td>4.2</td>
<td>858</td>
<td>30</td>
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<tr>
<td>7.</td>
<td>Second hydroxylapatite eluant</td>
<td>2,500</td>
<td>1.5</td>
<td>1670</td>
<td>21</td>
</tr>
</tbody>
</table>

* The direct assay (dihydroorotate formation from ureidosuccinate) was used.
Fig. 3. The elution profile from the first hydroxylapatite column.

Protein fractions were eluted using a linear potassium phosphate buffer (pH 6.8) gradient. Enzyme activity was determined by the increase in absorbance at 230 nm as given in Materials and Methods. Protein was estimated by the method of Warburg and Christian (28).
Protein fractions were eluted with a potassium phosphate buffer (pH 7.8) gradient. Peak B represents purified dihydroorotase with a specific activity of 1670 units/mg. Protein was estimated by absorbance at 220 nm using a standard curve of bovine serum albumin.
fraction at 4°C was found to be approximately 4 weeks unless a precipitate (probably ZnPO₄) occurred which usually initiated loss of activity. In general, the purification procedure indicated the dependence of dihydroorotase activity on an added cofactor, Zn²⁺. This data is consistent with that of Sander et al. (15), where partially purified enzyme was demonstrated to also be dependent upon Zn²⁺ or Co²⁺.

Figure 5 shows the results of analysis by polyacrylamide disc electrophoresis on the fractions from the purification procedure. All the gels are arranged so that the position of the enzyme protein coincide. Note that the purification was essentially complete after DEAE-Sephadex fractionation (no. 2) and the first hydroxylapatite chromatography (no. 3). Protein stain of the most purified fraction in 7.5% (no. 4) and 12% gels revealed a single homogeneous band. However, when high levels of protein were applied to the gel (100 ug), trace contaminant bands were observable. Detection of enzyme activity by slicing a 7.5% gel longitudinally down its axis and staining one-half the gel for protein while assaying 2 mm sections of the other half according to the procedure of Prescott and Jones (27) as given in Materials and Methods gave exact coincidence between the protein band and enzyme activity (Fig. 6).

Enzyme Properties

**Determination of pH optima** - The pH optima in both the biosynthetic and degradative directions were investigated (Figs. 7a and 7b). Dihydroorotase activity shows a marked dependence on pH. The biosynthetic reaction (Fig. 7a), the conversion of N-carbamyl-L-aspartate to L-dihydroorotate, shows a narrow optimum range from pH 5.8 to pH 6.2. There appears
Electrophoresis was done on 7.5% acrylamide columns. Migration was from bottom to top. All gels were stained for protein with Coomassie Brilliant Blue R as given in Materials and Methods. Fraction number used (see Table I): gel 1, fraction 1; gel 2, fraction 4; gel 3, fraction 6; gel 4, fraction 7.
Fig. 6. Comparison of dihydroorotase activity to protein stain of purified enzyme in polyacrylamide columns.

The activity in 2 mm gel slices was measured with L-dihydroorotate as the substrate. The product (N-carbamyl-L-aspartate) was detected by the procedure of Prescott and Jones (27) as outlined in the Materials and Methods.
Fig. 7a. The pH dependence of dihydroorotase catalyzed conversion of N-carbamyl-L-aspartate to L-dihydroorotate.

The reaction conditions are stated in Materials and Methods using 63 units of enzyme with a specific activity of 860 units/mg. Buffers: 67 mM MES-NaOH (○—○), 167 mM potassium phosphate (■—■), 167 mM Tris-HCl (□—□).
Fig. 7b. The pH dependence of dihydroorotase conversion of L-dihydroorotate to N-carbamyl-L-aspartate.

The reaction conditions are as stated in the Materials and Methods, using 31 units of enzyme with a specific activity of 860 units/mg. Buffers: 167 mM Tris-phosphate (●—●), 167 mM potassium phosphate (○—○), 167 mM glycine-NaOH (□—□).
to be little variation in activity at a particular pH due to differences in buffer composition, although MES-NaOH appears to stabilize the peak activity in the range from pH 5.8 to pH 6.0 much better than phosphate buffer. Dihydroorotase activity is negligible below pH 5.5 or above pH 7.8. From earlier studies on pH optima of crude extracts, the activity of the purified enzyme exhibits a much greater pH dependence since the biosynthetic reaction in crude preparations exhibits a broad peak of activity from pH 5.0 to 8.5.

The degradative reaction (Fig. 7b), the conversion of L-dihydroorotate to N-carbamyl-L-aspartate, exhibits a distinctly different pH dependence. Degradative dihydroorotase catalytic activity shows a broad optimum range from pH 7.5 to pH 8.5. It also appears to show higher levels of activity in the presence of phosphate buffer alone than in the presence of Tris-phosphate. The nature of this stimulation has not been presently investigated. Since the data indicates considerable enzyme activity in the very alkaline range, the possibility of non-enzymatic conversion of L-dihydroorotate to N-carbamyl-L-aspartate was eliminated by incubation of the substrate under identical conditions in the absence of enzyme.

In general, the degradative reaction exhibits a wider pH range of activity (pH 6.5 to pH 10.0) than observed with the biosynthetic reaction (pH 5.5 to pH 7.5). However, in the region defined as the center of optimal activity (pH 6.0 for the biosynthetic reaction and pH 8.2 for the degradative reaction) the competing reactions appear to be mutually exclusive of one another.
Influence of substrate concentration - Lineweaver-Burk plots of the dihydroorotase catalyzed conversion of N-carbamyl-L-aspartate to L-dihydroorotate, and L-dihydroorotate to N-carbamyl-L-aspartate are illustrated in Figures 8 and 9, respectively. The $K_m$ value for N-carbamyl-L-aspartate at pH 6.0 was 0.13 mM (Fig. 8), and for L-dihydroorotate at pH 8.2 was 0.07 mM (Fig. 9). No inhibitory effect of high substrate concentration was observed unless enzyme activity was allowed to decay to half of the original value. Using such decayed enzyme fractions a very distinct inhibition by high substrate concentration is observed in both the biosynthetic and degradative directions.

Molecular weight determination - The molecular weight of dihydroorotase was estimated by thin-layer gel filtration (Fig. 10). Considerable problems in locating the enzyme activity after gel filtration were eliminated when it was found necessary to make the eluting buffer 50 µM Zn$^{2+}$. From the basis of several runs the molecular weight of dihydroorotase was determined to be approximately 110,000.

Subunit composition - Native purified dihydroorotase was analyzed for subunits in sodium dodecyl sulfate polyacrylamide gels according to the procedures of Weber and Osborn (34) and Neville (35). Both systems were run twice yielding identical results. Plots of relative mobility of the molecular weight markers and enzyme to cytochrome c and brom phenol blue are shown in Figures 12 and 11, respectively. The monomer weight appeared to be approximately 56,000 daltons indicating that the native enzyme was composed of 2 subunits of identical molecular weight.
Fig. 8. Effect of N-carbamyl-D,L-aspartate concentration on L-dihydroorotate formation.

The reaction conditions are as stated in Materials and Methods using 68 units of enzyme with a specific activity of 860 units/mg in the presence of 67 mM MES-NaOH. The $K_m$ was calculated on the basis of N-carbamyl-L-aspartate.
Fig. 9. Effect of L-dihydroorotate concentration on N-carbamyl-L-aspartate formation.

The product was detected by the procedure of Prescott and Jones (27) as stated in the Materials and Methods using 31 units of enzyme with a specific activity of 860 units/mg in the presence of 167 mM Tris-phosphate buffer (pH 8.2).
Fig. 10. Estimation of the molecular weight of dihydroorotase using thin-layer gel filtration.

The procedure is as given in Materials and Methods using approximately 100 units of enzyme on a 20 cm thin-layer gel plate coated with Sephadex G-200 superfine (0.6 mm). Molecular weight markers: aldolase, 158,000; bovine serum albumin, 68,000; ovalbumin, 45,000; chymotrypsinogen, 25,000; ribonuclease A, 13,700.
Fig. 11. Determination of the subunit composition of dihydroorotase in sodium dodecyl sulfate polyacrylamide columns.

The procedure is given in Materials and Methods using the discontinuous buffer system of Neville (35). Mobility was determined relative to the migration of brom phenol blue. Molecular weight markers: bovine serum albumin, 68,000; catalase, 60,000; ovalbumin, 45,000; aldolase, 40,000.
Fig. 12. Determination of the subunit composition of dihydroorotase in sodium dodecyl sulfate polyacrylamide columns.

The procedure is outlined in Materials and Methods using the sodium dodecyl sulfate gel system of Weber and Osborn (34). Mobility was determined relative to the migration of cytochrome c. Molecular weight markers: bovine serum albumin, 68,000; ovalbumin, 45,000; pepsin, 35,000; trypsin, 23,000.
Atomic Absorption Spectroscopy - Purified dihydroorotase was analyzed for zinc content by atomic absorption spectroscopy according to the experimental details given in Materials and Methods. Figure 13 illustrates the protein elution pattern and zinc content of a Sephadex G-25 elution of concentrated purified enzyme. The protein to zinc concentration ratio was constant (within 11%) across the peak indicating the presence of zinc in the enzyme. An average value of 2 g atoms of zinc per 56,000 g of protein was found in the peak tube. Since the molecular weight of dihydroorotase is 110,000, it appears that the enzyme probably contains 4 zinc atoms per molecule of native dihydroorotase.
Fig. 13. Atomic absorption spectroscopy of purified dihydroorotase fractions from a Sephadex G-25 column.

Experimental details are listed in Materials and Methods. Protein concentration was estimated by the procedure of Lowry et al. (29).
DISCUSSION

The involvement of a metal ion cofactor in the degradative dihydro-orotase from *Cl. oroticum* has been suspected for some time, although definitive proof was lacking since no one had purified the enzyme to homogeneity and demonstrated the presence of Zn$^{2+}$ in the enzyme protein structure. Yates and Pardee in 1956 (12) during studies with extracts from *Cl. oroticum* cultured on glucose and orotate as sole sources of carbon and energy suggested that some unknown cofactor appeared to be involved in dihydroorotase activity. Evidence for that cofactor being either Zn$^{2+}$ or Co$^{2+}$ was indicated by Sander et al. (15) while attempting to purify degradative dihydroorotase from *Cl. oroticum*. They repeatedly observed a two to three fold decrease in specific activity during protamine sulfate and ammonium sulfate fractionation of the enzyme. Further studies showed that a heat-, acid-, and ultraviolet-stable cofactor was involved. Treatment of the enzyme with several metal chelators, particularly EDTA (which has good stability constants with divalent cations of transition elements) was shown to inhibit enzyme activity. Only Zn$^{2+}$ and Co$^{2+}$ served effectively to restore activity of EDTA-treated enzyme, Zn$^{2+}$ being the superior activator at low concentrations. This is quite reasonable since Zn$^{2+}$ and Co$^{2+}$ have similar ionic radii of 0.69 Å to 0.71 Å, and have been shown to effectively replace one another in other Zn$^{2+}$ containing metalloenzymes (40). Other metals tested included Fe$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, and Mn$^{2+}$. They failed to reactivate the enzyme above the control level, and in some cases inhibited the reaction rate. Sander et al. (15) were also able to demonstrate that it
was necessary to incubate the EDTA-treated dihydroorotase in the presence of Zn$^{2+}$ to promote maximal initial rates of enzyme activity, and to abolish the lag which is seen without incubation in the presence of Zn$^{2+}$. These results suggest that the Zn$^{2+}$ must first react with the enzyme to constitute the functional catalyst which then reacts with the substrate.

The present study supports the data concerning the dependence of dihydroorotase activity on Zn$^{2+}$. Streptomycin sulfate reactivation of the 150,000 x g extracts stored at -20$^\circ$C (see Materials and Methods) could be due to the addition of Zn$^{2+}$ present in the compound, since kinetic studies on active purified enzyme showed no effect of streptomycin sulfate on enzyme activity. Chromatography on DEAE-Sephadex in the presence of 0.5 mM Zn$^{2+}$ consistently showed a recovery of up to 150% of the original enzyme units, followed by a reduction to 60% of the original activity after extensive dialysis against 0.01 M potassium phosphate buffer (pH 6.8) which lacked Zn$^{2+}$. Hydroxylapatite chromatography followed by a 12 hour incubation in the presence of 50 μM Zn$^{2+}$ resulted in a two to three fold increase in specific activity of the enzyme. No increase was observed if the Zn$^{2+}$ was not added. Since hydroxylapatite has a strong affinity for such divalent metal cations as Zn$^{2+}$ and Co$^{2+}$ we could be observing loss of activity due to removal of Zn$^{2+}$ from the enzyme. However, definitive proof for Zn$^{2+}$ involvement in the protein structure comes from atomic absorption spectroscopy of the purified dihydroorotase.

Molecular weight and subunit analyses indicated that dihydroorotase has a molecular weight of approximately 110,000 and is composed of 2
identical subunits of 56,000 each. Using atomic absorption spectroscopy an average value of 2 g atoms of zinc per 56,000 g of protein was determined in the peak tube of a Sephadex G-25 elution of purified protein. This suggests that the native enzyme contains four zinc atoms per molecule of dihydroorotase. A comparison of this data to examples of other known zinc-containing enzymes such as carbonic anhydrase, carboxypeptidase, alkaline phosphatase, and alcohol dehydrogenase indicates that the normal ratio of Zn$^{2+}$ to protein structural units is 1:1 (zinc/unit) rather than 2:1 (zinc/unit) (40). Since it has been well-documented in this laboratory and others that sodium dodecyl sulfate treatment in the presence of reducing agent does not always completely break down an enzyme protein into its smallest components, it is quite tempting to speculate that the two 56,000 dalton subunits actually represent dimers which are very strongly bound together. So, dihydroorotase may be a tetramer consisting of 4 identical subunits, each containing a single zinc atom. It is further interesting to note that of the zinc-containing enzymes purified and characterized to date nearly all have similar catalytic functions: alkaline phosphatase involves the hydrolysis of many types of orthophosphate monoesters, carbonic anhydrase involves the reversible hydration of CO$_2$ to form HCO$_3^-$, and carboxypeptidase and dihydroorotase enhance the rate of hydrolysis of amide bonds which are in the immediate proximity of a carboxyl group. It appears that perhaps Zn$^{2+}$ plays a role in H$_2$O-substrate-enzyme interaction. Presently, dihydroorotase represents one of the more complex zinc-containing proteins which have been purified and partially characterized. The majority of zinc-containing proteins tend to be low molecular weight monomers or
dimers (40,000 daltons to 80,000 daltons), although alcohol dehydro-
genase is believed to be a tetramer involving four 20,000 dalton subunits with evidence for four zinc binding sites (40).

The metal requirement of the degradative dihydroorotase from Cl. oroticum is unique among the presently purified preparations of dihydro-
orotase from other sources. However, there are a number of similarities between it and the biosynthetic enzymes from E. coli B and pea plants (see Table II). Although $K_m$ values do differ considerably, all three purified enzymes exhibit a higher affinity for L-dihydroorotate than for N-carbamyl-L-aspartate. Sander et al. (15) reported a $K_m$ value for N-
carbamyl-L-aspartate for the partially purified degradative dihydroorotase of 0.8 mM, whereas the present paper reports a value of 0.13 mM. This discrepancy could be accounted for by the observation that their purifi-
cation procedure resulted in very poor recovery of enzyme activity, perhaps indicating some irreversible damage to the catalytic sites on the enzyme. Earlier work on cell-free extracts by Lieberman and Kornberg (9) with the degradative dihydroorotase gave a value of 0.28 mM which is in much closer agreement with data obtained in this laboratory using the purified enzyme. Another interesting similarity between the three enzymes is that the conversion of N-carbamyl-aspartate to dihydroorotate has a pH optimum in all the enzymes near pH 6.0, whereas the reverse reaction has a pH optimum in the region of pH 8.0. This correlates with the observation that the non-enzymatic conversion of dihydroorotate to N-carbamyl-aspartate is greatly enhanced under alkaline conditions (41). No further correlations between the biosynthetic and degradative dihydroorotases can be drawn due to lack of more detailed information.
<table>
<thead>
<tr>
<th></th>
<th>Degradative Dihydroorotase</th>
<th>Biosynthetic Dihydroorotase</th>
</tr>
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<tbody>
<tr>
<td>Cl. oroticum</td>
<td></td>
<td>E. coli B (16)</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>110,000</td>
<td>76,000</td>
</tr>
<tr>
<td>Subunit composition</td>
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<td></td>
</tr>
<tr>
<td>Km Value:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. N-Carbamyl-L-aspartate</td>
<td>0.13 mM</td>
<td>0.76 mM</td>
</tr>
<tr>
<td>2. L-Dihydroorotate</td>
<td>0.07 mM</td>
<td>-</td>
</tr>
<tr>
<td>pH optimum:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. N-Carbamyl-aspartate to Dihydroorotate</td>
<td>6.0</td>
<td>5.0</td>
</tr>
<tr>
<td>2. Dihydroorotate to N-Carbamyl-aspartate</td>
<td>8.2</td>
<td>8.5</td>
</tr>
<tr>
<td>Metal Ion Requirement</td>
<td>Zn$^{2+}$ or Co$^{2+}$</td>
<td>none</td>
</tr>
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<td></td>
<td>none</td>
<td>none</td>
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</table>
However, the unique presence of zinc in the degradative dihydroorotase could be of importance when considering the relationship between the inducible and constitutive enzymes in *Cl. oroticum*.

A close analysis of known data on orotate catabolism in certain bacterial species suggests several interesting possibilities concerning the relationship of inducible degradative dihydroorotase activity to constitutive biosynthetic dihydroorotase activity in these organisms. Orotate catabolism initially involves the catalytic activity of an enzyme dihydroorotate dehydrogenase (EC 1.3.3.1) which converts orotate to L-dihydroorotate (see Fig. 2). It has been purified from a number of sources and studied as a model iron-flavoprotein (42-46). The constitutive biosynthetic dihydroorotate dehydrogenase has been purified from *Lactobacillus bulgaricus* (47) and shown to differ from the inducible, degradative enzyme in *Cl. oroticum*. Taylor *et al.* (48) isolated a pseudomonad and demonstrated the presence of two different dihydroorotate dehydrogenases: a particle-bound constitutive biosynthetic enzyme formed when the *Pseudomonas* sp. was cultured on orotate, glucose, glycerol, or aspartate, and an inducible soluble degradative NADP-linked enzyme in addition to the biosynthetic enzyme when the organism was cultured on orotate. Also, the purification of the degradative dihydroorotate dehydrogenase from *Cl. oroticum* has been modified by Taylor and Eames (49), and a comparison of it to the biosynthetic enzymes from other sources indicates a large degree of dissimilarity. Thus, the evidence suggests the presence of two distinct dihydroorotate dehydrogenases catalyzing biosynthetic and degradative reactions in organisms capable of growing on orotate. Definitive proof of the two catalytically and
structurally different enzymes in *Cl. oroticum* is dependent upon purification of the constitutive biosynthetic dihydroorotate dehydrogenase which has been demonstrated to be present in the soluble fraction of glucose grown *Cl. oroticum* (49). Reflecting back to dihydroorotase, the evidence does suggest that an adaptive degradative dihydroorotase could also be different from the constitutive biosynthetic enzyme, especially because of its unique involvement of Zn$^{2+}$ in activity. Of course, there is the possibility that we are only observing higher levels of an single biosynthetic enzyme protein being produced when *Cl. oroticum* is cultured on orotate as the sole carbon and energy source.

One additional feature of this problem is a comparison of the structural similarities between the purified degradative dihydroorotase and the purified degradative dihydroorotate dehydrogenase from *Cl. oroticum* when isolated from identical cell-free extracts. During the course of the purification procedure it was observed that the migration distance on 7.5% polyacrylamide gels of the dihydroorotase is exactly coincident with that of the dihydroorotate dehydrogenase. Since criteria for homogeneity of an enzyme preparation is usually based on polyacrylamide gel electrophoresis, several other independent lines of evidence indicated that the dihydroorotase was free of dihydroorotate dehydrogenase. These included lack of dihydroorotate dehydrogenase activity and flavoprotein in purified preparations, homogeneous banding of dihydroorotase in 12% polyacrylamide gels, and different subunit banding patterns when purified dihydroorotate dehydrogenase and dihydroorotase were subjected to identical conditions with sodium dodecyl sulfate electrophoresis. Migration of proteins in acrylamide gels is dependent upon both molecular weight and charge. Since the reported molecular weight of the degradative dihydroorotate dehydro-
genase is 115,000 as compared to 110,000 for dihydroorotase, the similarity in migration in acrylamide gels must be due to similar characteristics in charge or amino acid composition. It is also interesting to note that not only does dihydroorotate dehydrogenase contain four Fe$^{2+}$, but also has a subunit composition of two 26,000 dalton and two 33,000 dalton subunits. If dihydroorotase is actually a tetramer consisting of 4 identical subunits (28,000 daltons each?) with 1 Zn$^{2+}$ per subunit, the similarity between the size of the subunits and charge on the enzymes suggest that perhaps dihydroorotase involves 4 subunits similar in composition to the two 26,000 subunits of the dihydroorotate dehydrogenase. Instead of Fe$^{2+}$ being the required cofactor responsible for either structural or catalytic properties, the protein involves Zn$^{2+}$. In terms of metabolic economy of the cell this describes an efficient organization with multiple use of a particular protein molecule for different catalytic functions. The evolutionary significance of this type of relationship is that it may be an example of gene duplication in the modification of an organism's capabilities for synthesizing and catabolizing various organic molecules.
SUMMARY

1. Dihydroorotase (4,5-L-dihydro-orotate amidohydrolase, EC 3.5.2.3) has been purified 102-fold from Clostridium oroticum cultured on orotate.

2. Molecular weight has been determined by thin-layer gel filtration to be approximately 110,000.

3. Subunit analyses show dihydroorotase to be composed of two identical subunits 56,000 daltons each.

4. Atomic absorption spectroscopy of the enzyme shows it to be a zinc-containing metalloenzyme with 4 g atoms of zinc per molecule of dihydroorotase.

5. The pH optima for the conversion of N-carbamyl-L-aspartate to L-dihydroorotate and L-dihydroorotate to N-carbamyl-L-aspartate have been determined to be at pH 6.0 and pH 8.2, respectively.

6. The binding constant of the enzyme for each substrate has been investigated with values of 0.13 mM for N-carbamyl-L-aspartate and 0.07 mM for L-dihydroorotate.
REFERENCES


49. Personal communication