Dihydroorotase from *Escherichia coli*

SULFHYDRYL GROUP-METAL ION INTERACTIONS* 

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We have obtained 53 mg of 99% pure dihydroorotase from 10.9 g of frozen *Escherichia coli* pyrC plasmid-containing *E. coli* cells using a 4-step 16-fold purification procedure, a yield of 60%. We characterize the enzyme by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (a dimer of subunit molecular weight 38,300 ± 2,900), high performance liquid chromatography gel sieving, amino acid analysis, amino terminus determination (blocked), and specific activity.

The isolated enzyme contains 1 tightly bound essential zinc atom/subunit, and readily but loosely binds 2 additional Zn(II) or Co(II) ions/subunit which modulate catalytic activity; treatment of crude extracts with weak chelators suggests that the enzyme contains 3 zinc atoms/subunit in vivo. Two of the 6 thiol groups/subunit react rapidly with 5,5'-dithiobis(2-nitrobenzoate) when 1 Zn/subunit enzyme is used, but slowly when 3 Zn/subunit enzyme is used. The 2 weakly bound Zn(II) ions/subunit protect against the reversible air oxidation which lowers the specific activity of the enzyme and renders it unreactive with 5,5'-dithiobis(2-nitrobenzoate).

The dilution activation observed in the presence of substrate, the dilution inactivation observed in the absence of substrate, and the transient activation by the metal chelator oxalate are interpreted as evidence for an unstable, hyperactive monomer.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Purification of pyrC Plasmid-derived *E. coli* Dihydroorotase—The purification of pyrC plasmid-derived *E. coli* dihydroorotase yielding 1 Zn/subunit enzyme is summarized in Table 1. This procedure provides a 16-fold purification with recovery of 60% of the activity found in the initial crude extract. The enzyme produced is 99% pure as judged by HPLC gel filtration and NH$_2$-terminal analysis (see below). The results shown in Table 1 are typical and reproducible. SDS-polyacrylamide gel electrophoretic analysis of each step from the enzyme purification is shown in Fig. 1; 5 μg of heavily stained protein from the last step gives a single band corresponding to an apparent subunit molecular weight of 38,300 ± 2,900 (n = 3). HPLC gel filtration of 10 mg of purified dihydroorotase gave a large symmetrical peak and two very small contaminating peaks which together represented less than 1% of the total combined area under all three peaks (data not shown). pyrC plasmid-derived 1 Zn/subunit dihydroorotase has a Michaelis constant ($K_m$) and turnover number ($k_{cat}$) for potassium dihydro-orotase at (pH 8.0) of 71.3 μM and 169 s$^{-1}$, respectively, is reasonable agreement with the values of 75.6 μM and 127 s$^{-1}$ obtained earlier for the ATCC 29477 chromosomally derived enzyme (1). The enzyme is stable for at least 2 months when stored under nitrogen at 4 °C in metal-free 10 mM N-carbamyl-DL-aspartate, 100 mM Tris phosphate buffer (pH 7.0).

**Amino Acid Composition and NH$_2$-terminal Analysis**—The amino acid composition of pyrC plasmid-derived *E. coli* dihydroorotase is shown in Table 2. The amino acid analyses for pyrC plasmid-derived and ATCC 29477 chromosomally derived (1) dihydroorotase agree quite well except for proline and cysteine. In our earlier determination (1), proline was inadvertently omitted and the value for cysteine was low. The enzyme contains 7.9 mol % aromatic and 51.9 mol % polar amino acid residues. The ($Axs + G lx$)/(Lys + Arg) ratio was 2.1, consistent with an acidic isoelectric point (1). A distinctive feature of the enzyme is the presence of only 2 tryptophan residues.

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TABLE 1
Purification of pyrC plasmid-derived E. coli dihydroorotase yielding 1 Zn/subunit enzyme

Starting material was 10.89 g of frozen pyrC plasmid-containing E. coli cell paste (2). An activity unit is defined as 1 μmol of dihydroorotase produced per min at 30 °C, pH 5.80, monitored by the increase in absorbance at 230 nm. The assay reaction was initiated from each purification step. Protein was determined by the method of Lowry et al. (15) as modified by Peterson (16).

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume Protein Units</th>
<th>Units/mg</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sonication</td>
<td>57 ml 1,410 33,100 mg</td>
<td>23.5 %</td>
<td>100 1</td>
<td></td>
</tr>
<tr>
<td>2. DEAE-cellulose</td>
<td>70 ml 390 35,400 mg</td>
<td>90.8 %</td>
<td>107 3.9</td>
<td></td>
</tr>
<tr>
<td>3. Heat step</td>
<td>65 ml 127 30,800 mg</td>
<td>243 93 10.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. DEAE-cellulose</td>
<td>43.5 ml 53.3 19,800 mg</td>
<td>371 60 15.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. SDS-polyacrylamide gel electrophoretic analysis of each step from the purification of pyrC plasmid-derived E. coli dihydroorotase. A 10–20% linear gradient polyacrylamide gel was run in SDS according to the method of Laemmli (21). Lanes A and H contained marker proteins: phosphorylase b (Mr = 94,000), bovine serum albumin (Mr = 67,000), ovalbumin (Mr = 43,000), carbonic anhydrase (Mr = 30,000), soybean trypsin inhibitor (Mr = 20,100), and α-lactalbumin (Mr = 14,400). Lanes C through F contained samples of dihydroorotase (1.9 units): lane C, step 1, 79 μg; lane D, step 2, 20.4 μg; lane E, step 3, 7.7 μg; lane F, step 4, 5.0 μg. Lanes B and G contained no protein. Units of dihydroorotase were determined in the intact protein using DTNB; see Fig. 7.

residues/subunit. The subunit molecular weight, calculated assuming 341 amino acids/subunit, is 38,050, in good agreement with the value determined by SDS–polyacrylamide gel electrophoresis (38,300) and previous work (1).

The native molecular weight for ATCC 29477 chromosomally derived dihydroorotase reported earlier (1) was determined by sedimentation equilibrium, which requires an accurate value for the partial specific volume. The partial specific volume of the pyrC plasmid-derived enzyme, computed from the amino acid composition reported here, is 0.732 ml/g at 20 °C, which differs from the value calculated previously for the ATCC 29477 chromosomally derived enzyme by –0.7% (1). The native molecular weight for dihydroorotase is 75,000 ± 5,700 (n = 4) when recalculated using the partial specific volume of the pyrC plasmid-derived enzyme and the sedimentation equilibrium data from the ATCC 29477 chromosomally derived enzyme (1).

Analysis using a protein-peptide sequencer revealed only 1 mol % of the expected free NH₂ termini in the purified pyrC plasmid-derived dihydroorotase. We conclude that the dihydroorotase purified as described in Table 1 has a blocked NH₂ terminus.

Metal Content of Dihydroorotase—Dihydroorotase, purified as described in Table 1, contains one equivalent of tightly bound Zn/subunit after extensive dialysis against a chelating resin under conditions in which the enzyme is stable and active. Dialysis of 1 Zn/subunit dihydroorotase against ZnCl₂ or CoCl₂ and then against a chelating gel added two metal ions/subunit (determined by atomic absorption spectroscopy) and altered the kinetic properties of the enzyme (Table 3). Dialysis against the much larger Mn(II) ion (as MnCl₂) and
then against a chelating gel did not add metal ions to the enzyme. Dialysis of the fully metallated enzyme at pH 7.0 against N-carbamyl-DL-aspartate did not remove either Zn or Co(II) ions from the weak sites. However, dialysis of the fully metallated enzyme at pH 5.80 against N-carbamyl-DL-aspartate removed the two additional Zn ions, but not the Co(II) ions from the weak sites. Oxalate removal at pH 5.30 of the Zn from 1 Zn/subunit dihydroorotase produces inactive apoenzym. Air-oxidized 1 Zn/subunit dihydroorotase did not bind additional Zn or Co(II). 3 Zn and 1 Zn, 2 Co(II)/subunit dihydroorotase are stabilized relative to 1 Zn/subunit dihydroorotase against activity loss by air oxidation (Fig. 2).

Dilution Activation of Dihydroorotase—When the concentrated extract obtained from each purification step was assayed over a wide range of different enzyme concentrations (5–2000 ng/ml dihydroorotase, or 0.1–50 nM in active sites), the specific activity was found to increase at least 2-fold as the enzyme concentration decreased below about 50 ng/ml if substrate was always present during the process of diluting the enzyme into the assay mix (Table 4, Miniprint).

Dilution Inactivation of 1 Zn and 3 Zn/subunit Dihydroorotase—Dihydroorotase is inactivated upon dilution below a protein concentration of about 100 μg/ml (Fig. 3), and the time dependence of this inactivation for 1 Zn and 3 Zn/subunit dihydroorotase at an enzyme concentration of 100 μg/ml is shown in Fig. 4. Activity loss was first order, with coefficients of determination greater than 0.99, as long as the samples were incubated under nitrogen. 1 Zn/subunit dihydroorotase is more susceptible to dilution inactivation than 3 Zn/subunit dihydroorotase. The half-lives for inactivation of 1 Zn and 3 Zn/subunit dihydroorotase are 10.2 and 21.7 h, respectively, using the conditions indicated.

The temperature dependence of the dilution inactivation of 1 Zn/subunit dihydroorotase is shown in Fig. 5, an Arrhenius plot (18) of the first-order rate constant for inactivation obtained at several incubation temperatures. The coefficient of determination of the linear least squares fit is 0.98. The activation energy for the thermal inactivation process under these incubation conditions, calculated from the slope of this plot, is 4.8 kcal/mol. The half-life for 1 Zn/subunit dihydroorotase activity loss was 4.5 h at 0 °C and 1.7 h at 36.5 °C, using the conditions indicated. Since no temperature of maximum enzyme stability was observed in the range of 0–36.5 °C, dihydroorotase does not appear to be cold-labile (23). First-order kinetics were not observed if the dihydroorotase samples were exposed to air during the incubation: maintenance of a nitrogen atmosphere was required to avoid activity loss from air-oxidation.

Oxalate-mediated Activation-Inactivation of 1 Zn and 3 Zn/subunit Dihydroorotase—The chelator oxalate has a biphasic effect on the specific activity of dilute 1 Zn and 3 Zn/subunit dihydroorotase as a function of time (Fig. 6). A relative specific activity of 1.0 was assigned to the initial activity of 1 Zn/subunit dihydroorotase in the absence of oxalate. 1 Zn and 3 Zn/subunit enzyme were both activated by oxalate to a relative specific activity of 2.0, even though their initial specific activities were very different (1.0 and 0.07, respectively). However, 3 Zn/subunit dihydroorotase was completely activated within 5 min, whereas 1 Zn/subunit dihydroorotase required 6 h for maximum activation. The inactivation rate from the activity maximum was also quite different for 1 Zn and 3 Zn/subunit dihydroorotase. 3 Zn/subunit enzyme activity decayed with a half-life of approximately 6 h, whereas inactivation of 1 Zn/subunit enzyme occurred with a half-life of about 2 days.

Reaction of 1 Zn and 3 Zn/subunit Dihydroorotase with DTNB—The time course for reaction of dihydroorotase with DTNB at pH 7.0 and 30 °C in the presence and absence of

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3 The metal ion at the tight site is given first; those at the weak sites are given second.
**Dihydroorotase from E. coli**

**Fig. 4.** Dilution inactivation of 1 Zn and of 3 Zn/subunit dihydroorotase: Time dependence. 1 Zn (○) and 3 Zn/subunit (●) dihydroorotase (106 μg/ml) in 10 mM bis-tris propane acetate buffer (pH 6.64) were incubated in the absence of substrate at 30 °C under nitrogen. Dihydroorotase activity loss was followed using potassium dihydro-DL-orotate as substrate as described under “Experimental Procedures.” The dilution inactivation data are plotted as log10 (dihydroorotase activity remaining) versus hours of incubation at 30 °C. First-order curves with coefficients of determination greater than 0.99 were obtained as long as the samples were incubated under a nitrogen atmosphere. The first-order rate constants for inactivation of 1 Zn and 3 Zn/subunit dihydroorotase are 0.065 and 0.032 h⁻¹, respectively.

Guanidine hydrochloride is shown in Fig. 7. With denatured enzyme, six thiol groups/subunit react instantaneously, in accord with the amino acid composition (Table 3). In the absence of deaturant for either 1 Zn or 3 Zn/subunit dihydroorotase, only 2 thiol residues/subunit react: no further reaction occurs during prolonged incubation of the enzyme with DTNB under the conditions specified. However, enzyme more dilute than 150 μg/ml frequently showed more than two thiols reacting/subunit (data not shown), apparently caused by disaggregation of the dimer and unfolding of the monomers. The rate of reaction of native 3 Zn/subunit dihydroorotase with DTNB is much less than that for native 1 Zn/subunit enzyme.

Kinetic analysis of these two reactive thiols/subunit in the presence of a 50-fold molar excess of DTNB (Fig. 8) indicates that the reaction for 3 Zn/subunit dihydroorotase can be defined by a single rate process (kobs = 0.0047 s⁻¹ at pH 7.0, μ = 0.015, 30 °C). However, for 1 Zn/subunit dihydroorotase the two thiol groups react at distinctly different rates with DTNB. A pseudo-first-order plot of the rate of reaction with excess DTNB is biphasic (Fig. 8), indicating the presence of two distinct classes of thiol groups with different reactivities toward DTNB. The apparent first-order rate constant for the slow phase of the reaction is computed to be 0.011 s⁻¹ (pH 7.0, μ = 0.015, 30 °C). Using this value to correct the experimental results at early times yields a rate constant of kobs = 0.25 s⁻¹ at pH 7.0, μ = 0.015, 30 °C, for the fast phase of the reaction. The total number of reacting thiols is two (Fig. 7).

Since the number of slow reacting thiols equals the number of fast reacting thiols (see the y intercept of panel B in Fig. 8), there must be one slow reacting thiol and one fast reacting thiol/subunit for 1 Zn/subunit enzyme. Removal of the two nonessential Zn ions from 3 Zn/subunit dihydroorotase to form 1 Zn/subunit enzyme results, as expected, in biphasic thiol reactivity with DTNB.

The pseudo-first-order rate constants for reaction of the two reactive thiols in 1 Zn/subunit dihydroorotase are linearly dependent on the DTNB concentration, as shown in Fig. 9. The coefficient of determination of the linear least squares fit is greater than 0.99 for both thiols. The second-order rate constants are 2450 and 137 M⁻¹ s⁻¹ for the fast and slow reacting thiols, respectively. Thus, there is no apparent saturation effect over the DTNB concentration range studied and no evidence for noncovalent complex formation between dihydroorotase and DTNB prior to modification of either thiol.

Formation of the dihydroorotase 2-nitro-5-thiobenzoic acid mixed disulfide lowers the specific activity of the enzyme by 50%; full activity returns immediately upon reduction with excess 2-mercaptoethanol.

The substrate N-carbamyl-DL-aspartate bound at the active site does not alter significantly the reactivity of either thiol with DTNB in 1 Zn, or 3 Zn/subunit dihydroorotase. Air-oxidized 1 Zn/subunit dihydroorotase does not react with DTNB: only 14% of the two reactive thiols reacted during a 30-min incubation of the enzyme with DTNB at pH 7.0 and 30 °C.

**Fig. 5.** An Arrhenius plot for the dilution inactivation of 1 Zn/subunit dihydroorotase. 1 Zn/subunit dihydroorotase (13.4 μg/ml) in 2 mM Tris phosphate buffer (pH 7.0) was incubated under nitrogen at 0, 3.5, 10, 16.5, 22, 30, and 36.5 °C. Dihydroorotase activity loss over a 12-h period for each temperature was followed using potassium dihydro-DL-orotate as substrate, and first-order rate constants for activity loss were calculated as described under “Experimental Procedures.” The activation energy for the thermal inactivation process, calculated from the slope of this plot, is 4.8 kcal/mol. The coefficient of determination of the linear least squares fit is 0.98.

**Comparison of Sonicated Dihydroorotase as a Crude Extract and as a Pure Enzyme**—A comparison of the relative activity of sonicated E. coli dihydroorotase in crude extracts with that of the sonicated purified enzyme is shown in Fig. 10. Sonication of crude extracts of E. coli cells in the absence of added chelators, or sonication of purified 3 Zn/subunit dihydroorotase in the absence of added chelators produces enzyme which has not yet been activated. Sonication of crude extracts of or of
purified 3 Zn/subunit dihydroorotase with weak metal chelators such as phosphate or N-carbamyl-DL-aspartate at pH 5.8 or 7.0 activates to the level expected for the 1 Zn/subunit enzyme; treatment with Bio-Rex 70 at pH 7.0 has the same effect. Sonication of purified 1 Zn/subunit dihydroorotase with or without added phosphate or N-carbamyl-DL-aspartate does not alter its relative activity.

Enzyme Purification—The enzyme purification reported here for pyrC plasmid-derived E. coli dihydroorotase is a simplification of our earlier purification for the ATCC 29477 chromosomally derived enzyme (1) and requires the same scrupulously clean solutions. This procedure is easy to scale up, and should yield grams of enzyme with little difficulty. Buffer preparation requires about 3 days and the enzyme purification about 5 days. Cloning the E. coli pyrC gene seems not to have altered the structure of dihydroorotase: the peptide maps of the pyrC plasmid-derived and ATCC 29477 chromosomally derived enzymes are indistinguishable (2), the subunit molecular weights are identical within experimental error (1), and the specific activities are similar (that of the plasmid-derived enzyme being about 33% higher; we believe that the HPLC gel sieving step in our earlier procedure (1) caused partial oxidation of the enzyme). The enzyme was particularly susceptible to dilution inactivation (e.g., see Fig. 3) when it was available only in small quantities, and required organic solutes such as ethylene glycol (9) for stabilization; such stabilizers are no longer required in the purification or handling of E. coli dihydroorotase. The blocked NH$_2$ terminus of the plasmid-derived enzyme which is purified in the absence of organic solutes indicates that the NH$_2$ terminus is blocked in vivo and not through modification by derivatives of organic stabilizers, such as aldehydes (1, 9). The native molecular weight of dihydroorotase of 75,000 ± 5,700 determined here agrees well with that of Sander and Heeb (24) obtained from Sephadex G-200 column chromatography (75,000 ± 10%).

Enzyme Specific Activity and Stability—E. coli dihydroorotase is a dimer of subunit molecular weight 38,400 (1), and, as isolated, contains one tightly bound Zn ion/subunit. This tightly bound Zn ion is probably at the active site because (a) its removal causes inactivation (Table 3), (b) its replacement with Co(II) produces a hyperactive enzyme, (c) all well-characterized dihydroorotase-like enzymes contain Zn (25–27), and (d) there is an obvious chemical role for the

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4 M. W. Washabaugh and K. D. Collins, unpublished experiments.
5 The dihydroorotase domain of the multifunctional mammalian carbamyl phosphate synthetase-aspartate transcarbamylase-dihydroorotase protein has been shown to contain one zinc ion (59).
Zn in the catalytic mechanism, as illustrated by the Zn(II)-
containing amidohydrolase carboxypeptidase A (28).

Several factors affect enzyme specific activity and stability: 
(i) the sulfhydryl oxidation state, (ii) the state of subunit 
aggregation, (iii) the presence and type of loosely bound metal 
ions, and (iv) the presence of a tightly bound metal ion. These 
phomena are summarized in a tentative structural model 
for the enzyme in Fig. 11.

Sulfhydryl Group-Metal Ion Interactions—Fig. 10 suggests 
that the two external metal binding sites of E. coli dihydroor-
otase are occupied in crude extracts, and thus also in vivo. 
Removal of Co(II) from the two external metal binding sites 
lowers the specific activity of the pure enzyme, whereas re-
moval of Zn(II) from the two external metal binding sites 
raises the specific activity of the pure enzyme (Table 3). Since 
the crude extract is activated by sonication in the presence of 
added weak chelators, the external metal ions in vivo are probably Zn(II). Although the specific activity of the sonicated crude 
extract without added chelators is somewhat higher than that 
for the sonicated 3 Zn/subunit dihydroorotase without added 
chelators (Fig. 10), the sonicated crude extracts, which contain 
low levels of endogenous chelators, may be a mixed population 
of less active 3 Zn and more active 1 Zn/subunit dihydrooro-
tase, resulting in a slightly elevated specific activity relative 
to 3 Zn/subunit dihydroorotase.

Protons compete with metal ions for Lewis base binding 
sites on enzymes: the ability of the weak chelator N-carbamyl-
dl-aspartate to remove the two external Zn ions at pH 5.8 
but not at pH 7.0 (in the absence of sonication) shows the 
expected pH dependence.

In the presence of the two loosely bound Zn ions, two of 
the six enzyme sulfhydryls/subunit react slowly with DTNB, 
and this reaction can be defined by a single rate process. The 
removal of these two loosely bound Zn ions increases the 
reactivity of one sulfhydryl with DTNB by a factor of 2.3 and 
the reactivity of the other sulfhydryl by a factor of 53. There 
is thus a different rate-limiting step at pH 7.0 for DTNB 
modification of the two reactive thiols when loosely bound 
metals are present: release of metal and exposure of the thiols 
to DTNB is probably rate-limiting. The two loosely bound 
metal ions also stabilize the two reactive sulfhydryls against 
air oxidation (Fig. 2).

Air oxidation of dihydroorotase results in a stable enzyme 
of decreased specific activity and is the major problem in 
handling this enzyme. The enzyme is stable to air oxidation 
if trace metal ions are carefully removed and the solutions are 
degassed to a dissolved O2 content of about 12 μl; that is, to 
about 20% that of an air-equilibrated solution (1). The specific 
activity of the air-oxidized enzyme ranges from 25 to 60% of 
that for the reduced enzyme, depending on enzyme purity and 
the trace metal content of the buffer during the oxidation. 
Air-oxidized 1 Zn/subunit dihydroorotase does not react with 
DTNB, which implies the oxidation of the same two thiols/
subunit, and does not bind additional metal ions, which 
implies the presence of one DTNB reactive sulfhydryl at each 
of the two external metal binding sites. The oxidative modi-
fication is reversible with dithioerythritol, restoring full en-
zyme activity and the DTNB reactivity of two thiols. The 
reversibility by thiols of the oxidative modification, and the 
loss upon oxidation of reactivity with the highly selective thiol 
reagent DTNB (29) suggests that cysteine is being oxidized 
to either a sulfenic acid (an intermediate in the formation of 
disulfides (30) previously detected in proteins (31, 32)), or a 
disulfide. A sulfenic acid has been drawn in Fig. 11 because 
there appears to be one reactive thiol for each separate metal 
binding site.

The structure-reactivity correlations for the reaction of 
simple thiol anions with DTNB predict a second-order rate
Dihydroorotase from E. coli

constant of approximately 200,000 M$^{-1}$ s$^{-1}$ for the reaction of DTNB with an unhindered thiol anion (where $pK_a$ (thiol) = 8.5) in aqueous solution at pH 7.0 and 30°C (7). The second-order rate constants for reaction of DTNB with the two thiols on 1 Zn/subunit enzyme are 137 and 2,450 M$^{-1}$ s$^{-1}$ at pH 7.0 and 30°C. Thus, the two reactive thiols are approximately

1,500- and 80-fold less reactive than an unhindered thiol anion, respectively. These second-order rate constants for the modification of 1 Zn/subunit dihydroorotase by DTNB are typical: second-order rate constants for the modification of protein thiols with DTNB range from 11 M$^{-1}$ s$^{-1}$ for Lactobacillus 30a histidine decarboxylase (33) to 3,460 M$^{-1}$ s$^{-1}$ for bovine seminal ribonuclease (34) at neutral pH. Human 5-

aminovalerate dehydrogenase has two thiol groups whose activity with DTNB is influenced by the presence of Zn ions in a manner similar to dihydroorotase (35). Changes in enzyme activity after metal substitution at external sites have also been observed by others. For example, the type of ion occupying the activation site in bovine lens leucine aminopeptidase strongly influences the maximum velocity of the reaction, although it has little effect on the Michaelis constant (36).

Evidence for an Unstable, Hyperactive Monomer—In Fig. 11 we interpret the activity changes which occur at low enzyme concentrations to dissociation of the dimer to an unstable, hyperactive monomer. This monomer is moderately stable at low concentrations when substrate is present; thus an increase in specific activity is initially observed upon dilution in the presence of substrate. This monomer is unstable at low concentrations when substrate is absent, and unfold; thus a decrease in enzyme specific activity is observed upon dilution in the absence of substrate.

In Fig. 12 we estimate that the specific activity of the hyperactive monomer is about 4 times that of a subunit in the 1 Zn/subunit dimer. Since oxalate activation occurs more rapidly with the 3 Zn/subunit enzyme than with the 1 Zn/subunit enzyme, oxalate may be removing the tightly bound Zn ion faster from 3 Zn than from 1 Zn/subunit enzyme, suggesting differences at the active site of the 3 Zn/subunit enzyme caused by the two external metal ions. Although the oxalate activation of 1 Zn and 3 Zn/subunit enzyme to the same level suggests the production of similar enzyme forms from either starting material, the fact that oxalate inactivates these intermediate enzyme forms at different rates depending upon the initial external metal content suggests that the hyperactive monomer derived from 3 Zn/subunit dimer keeps its distinguishing property: the external metal ions. Oxalate may be incapable of removing the two external Zn ions from the protein while having a specific affinity for the essential Zn ion because of the particular characteristics of the enzyme active site. This interpretation requires that the two external Zn ions/subunit, which lower $K_m$ of the dimer (Table 3), have no such effect on the isolated monomer.

Beckwith et al. (37) found that EDTA stimulated the activity of dihydroorotase in crude extracts up to 5-fold, whereas citrate and 1,10-phenanthroline were not as effective. EDTA binds Zn(II) tightly (log stability constant = 16.4) whereas oxalate binds Zn(II) weakly (log stability constant = 4.9) (38). We have not yet examined the effect of chelators other than

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4 In Table 3, oxalate extraction of the active site Zn from 1 Zn/subunit dimer was done in the presence of the stabilizer inositol to keep the apoenzyme from unfolding (see "Experimental Procedures").
oxalate on the purified enzyme. Since the monomer appears to be active, we conclude that all catalytic residues at the active site are contributed by the same polypeptide chain.

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REFERENCES

SUPPLEMENTARY MATERIAL TO
Dihydroorotase from Escherichia coli

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EXPERIMENTAL PROCEDURES

MATERIALS: All water was H2O-2 grade and was prepared on a 4 column Milli-Q water system including a Gencal-1 cartridge (Millipore Corp.). All chemicals were analytical or research grade. Thio-2,4 dinitrophenylhydrazine (DNPH) was obtained from Calbiochem (La Jolla, CA). Dihydroorotase (2,4-dihydroxy-L-serine, yielding L-isoleucine) was a gift from Dr. John K. Johnston (University of California, San Francisco). All reagents were stored at -20°C.

METHODS: Dihydroorotase was purified from E. coli K-12 strain B (ATCC 11377) growing in L-broth. The cultures were harvested by centrifugation (2500 x g, 15 min) and washed with 2.5 ml of 100 mM NaCl containing 0.5 M MnCl2. The cells were broken by sonication for 5 min at 150 W, and the sonicated cell-free supernatant was centrifuged at 105,000 x g for 60 min to remove cell debris. The supernatant was dialyzed against 50 mM potassium phosphate buffer (pH 7.5) at 4°C for 16 h.

The precipitate that forms during dialysis was not removed before step 1.

RESULTS: Dihydroorotase from E. coli K-12 strain B (ATCC 11377) was purified from E. coli K-12 strain B (ATCC 11377) growing in L-broth. The cultures were harvested by centrifugation (2500 x g, 15 min) and washed with 2.5 ml of 100 mM NaCl containing 0.5 M MnCl2. The cells were broken by sonication for 5 min at 150 W, and the sonicated cell-free supernatant was centrifuged at 105,000 x g for 60 min to remove cell debris. The supernatant was dialyzed against 50 mM potassium phosphate buffer (pH 7.5) at 4°C for 16 h.

The precipitate that forms during dialysis was not removed before step 1.

PROTEIN DEPENDENCE: Proteins were determined by the method of Lowry et al. (1951) as modified by Peterson (1977) with BSA as the standard. Interfering substances were removed using the phenylisothiocyanate-EDTA precipitation procedure described by Peterson (1977). The spectrophotometric protein assay was calibrated for dihydroorotase by amino acid analysis (10) and 2,4-dihydroxy L-serine. The protein content results for dihydroorotase were determined using the Bio-Rad dye binding method (10).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: HPLC gel filtration was performed as previously described (13). The protein elution profiles were monitored by absorbance at 280 nm using a full scale absorbance range of 0.01 a.

CYTOCHROME SPECTROSCOPY: Cytochrome c samples (approximately 100 μg/ml) were analyzed for size (1.3 mg, detection limit 5 μl), absorbance (280 nm, detection limit 1 μg) using a Varian Spectrophotometer. Absorbance at 280 nm was measured using a 1 cm cuvette at 25°C. Background signal was subtracted for all wavelengths using a 1 cm cuvette at 25°C. The specific activity of the enzyme was calculated using the formula (A280 - B280) x dilution factor / enzyme units.

PREPARATION OF ENZYMATICALLY-INDUCED DIHYDROOROTASE: E. coli strain B (ATCC 11377) was grown in L-broth at 37°C for 16 h, and the cells were harvested by centrifugation (2500 x g, 15 min). The crude extract was assayed for dihydroorotase activity.

METAL DETECTION AT THE LIMITED LEVELS: E. coli strain B (ATCC 11377) was grown in L-broth at 37°C for 16 h, and the cells were harvested by centrifugation (2500 x g, 15 min). The crude extract was assayed for dihydroorotase activity.

COMPLETE METAL DETECTION FROM DIHYDROOROTASE: E. coli strain B (ATCC 11377) was grown in L-broth at 37°C for 16 h, and the cells were harvested by centrifugation (2500 x g, 15 min). The crude extract was assayed for dihydroorotase activity.

Dihydroorotase from E. coli

COMPARISON OF DIHIDROOROTASE AS A CROUSE EXTRACT AND AS A PURE PREPARED DIHIDROOROTASE ACTIVITY IN ALL SAMPLES WAS DETERMINED USING POTASSIUM DIHYDRO-ORO-0-ROTRAN AS THE SUBSTRATE.

a: Preparation of CROUSE Extract by Disassembly: Dihydroorotase of an undefined metal content was obtained on a crude enzyme extract from yeast glycogen-dissociating S. pasteurii (1) by suspending 2 g of freeze-dried powder in 20 ml of 0.5M tris-HCl-potassium acetate buffer (pH 7.2, 25°C) in the presence of or absence of 25 mM E. coli (strain O1:K1) (serum) in a 35-ml glass beaker, and proceeding at 25°C as described in step 1 (above) for the yeast glycogen-dissociated enzyme purification procedure. Two 2-ml Biuret test (tris-HCl) to the suspension and 90 ml of buffer was added to the suspension or absence of 25 mM E. coli (strain O1:K1) (serum) in a 35-ml glass beaker, and the suspension was collected and 10% of buffer and the Biuret was collected as one fraction (2.2 ml).

b: Preparation of Pure Prepare: Purified 1.2 or 3 mM dihydroorotase (170 and 250 mg/mo, respectively) in 50 ml of 0.2M potassium acetate buffer (pH 7.2, 25°C) in the presence or absence of 25 mM E. coli (strain O1:K1) (serum) in a 35-ml glass beaker, and the suspension was collected as one fraction (2.2 ml). The Biuret was collected as one fraction (2.2 ml). The Biuret was collected as one fraction (2.2 ml). The Biuret was collected as one fraction (2.2 ml). The Biuret was collected as one fraction (2.2 ml).

### Table A

**Dihydroorotase activity of S. pasteurii dihydroorotase in the presence of substrate**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>0.001</th>
<th>0.01</th>
<th>0.1</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 mg/ml</td>
<td>2.8</td>
<td>3.1</td>
<td>3.2</td>
<td>3.3</td>
</tr>
<tr>
<td>0.1 mg/ml</td>
<td>2.7</td>
<td>2.9</td>
<td>3.0</td>
<td>3.1</td>
</tr>
<tr>
<td>0.01 mg/ml</td>
<td>2.6</td>
<td>2.8</td>
<td>2.9</td>
<td>3.0</td>
</tr>
<tr>
<td>0.001 mg/ml</td>
<td>2.5</td>
<td>2.6</td>
<td>2.7</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Specific activity relative to pure enzyme**

- **Concentration**
  - 1.0 mg/ml: 2.8
  - 0.1 mg/ml: 2.7
  - 0.01 mg/ml: 2.6
  - 0.001 mg/ml: 2.5

**Evidence for the Yeast glycogen-dissociated enzyme as a preparation**

- The yeast glycogen-dissociated enzyme was produced using purification steps previously described (1) for the yeast glycogen-dissociated enzyme.

**Evidence for the Yeast glycogen-dissociated enzyme as a preparation**

- The yeast glycogen-dissociated enzyme was produced using purification steps previously described (1) for the yeast glycogen-dissociated enzyme.