Divalent Metal Derivatives of the Hamster Dihydroorotase Domain†

Danny T. C. Huang, Michael A. W. Thomas, and Richard I. Christopherson*

Department of Biochemistry, University of Sydney, Sydney, New South Wales 2006, Australia

Received April 14, 1999; Revised Manuscript Received June 11, 1999

ABSTRACT: Dihydroorotase (DHOase, EC 3.5.2.3) is a zinc enzyme that catalyzes the reversible cyclization of *N*-carbamyl-L-aspartate to L-dihydroorotate in the third reaction of the de novo pathway for biosynthesis of pyrimidine nucleotides. The recombinant hamster DHOase domain from the trifunctional protein, CAD, was overexpressed in *Escherichia coli* and purified. The DHOase domain contained one bound zinc atom at the active site which was removed by dialysis against the chelator, pyridine-2,6-dicarboxylate, at pH 6.0. The apoenzyme was reconstituted with different divalent cations at pH 7.4. Co(II)-, Zn(II)-, Mn(II)-, and Cd(II)-substituted DHOases had enzymic activity, but replacement with Ni^{2+}, Cu^{2+}, Mg^{2+}, or Ca^{2+} ions did not restore activity. Atomic absorption spectroscopy showed binding of one Co(II), Zn(II), Mn(II), Cd(II), Ni(II), or Cu(II) to the enzyme, while Mg(II) and Ca(II) were not bound. The maximal enzymic activities of the active, reconstituted DHOases were in the following order: Co(II) → Zn(II) → Mn(II) → Cd(II). These metal substitutions had major effects upon values for V_{max}; effects upon the corresponding K_m values were less pronounced. The pK_a values of the Co(II)-, Mn(II)-, and Cd(II)-substituted enzymes derived from pH−rate profiles are similar to that of Zn(II)−DHOase, indicating that the derived pK_a value of 6.56 obtained for Zn−DHOase is not due to ionization of an enzyme−metal aquo complex, but probably a histidine residue at the active site. The visible spectrum of Co(II)-substituted DHOase exhibits maxima at 520 and 570 nm with molar extinction coefficients of 195 and 210 M^{-1} cm^{-1}, consistent with pentacoordination of Co(II) at the active site. The spectra at high and low pH are different, suggesting that the environment of the metal binding site is different at these pHs where the reverse and forward reactions, respectively, are favored.

Dihydroorotase (DHOase)[1] catalyzes the reversible cyclization of *N*-carbamyl-L-aspartate (CA-asp) to L-dihydroorotate (DHO) in the third reaction of the pathway for the de novo biosynthesis of pyrimidine nucleotides.

$$\text{CA-asp}^{2-} + \text{H}^{+} \leftrightarrow \text{DHO}^{-} + \text{H}_2\text{O} \qquad (1)$$

In prokaryotes, protozoa, and yeast, the enzyme is expressed as a distinct polypeptide. In higher eukaryotes, it is part of a trifunctional protein called CAD, which consists of the first three enzymes of the pyrimidine pathway in the sequence of H_3N^+−CPSase−DHOase−bridge−ATCase−COO^- (*1*−*3*). The DHOase domain has been purified from hamster CAD digested with elastase and found to be an 88 kDa dimer containing one tightly bound zinc atom per subunit (*4*). Time-dependent inactivation of murine DHOase with L-cysteine and analogues suggested that there was a zinc atom bound at the active site (*5*). The monofunctional DHOase from *Clostridium oroticum* contains two zinc atoms per monomer (*6*), while DHOase from *Escherichia coli* contains one zinc at the active site and two loosely bound structural zincs, which are not essential for activity (*7*). Removal of zinc from the DHOases found in *C. oroticum* (*8*, *9*) and *E. coli* (*10*) abolished enzymic activity which could be restored with Zn^{2+}, consistent with zinc bound at the active site with a catalytic role.

Site-directed mutagenesis of amino acid residues conserved between DHOases from different species has provided some information about the active site and the mechanism of catalysis (*11*, *12*). For the hamster DHOase domain, His15 and His17 have been identified as zinc ligands and the third ligand has been proposed to be His134 or His158 (*11*, *12*).[2] Arg19 and Lys239 were proposed to be involved in substrate binding, possibly by forming electrostatic and hydrogen bonds, respectively, with the substrate. Asp230 may act as a general acid or base in catalysis (*12*). His234 was proposed to be at the active site and to participate in catalysis (*11*). Asp13, His15, and zinc were proposed to form a C═O··· His → Zn^{II} triad, where the carboxylate of Asp13 contributes the carbonyl group of the triad. This motif is commonly found in zinc enzymes such as carbonic anhydrase (EC 4.2.1.1) and adenosine deaminase (EC 3.5.4.4) where

† This research was supported by an Institutional Grant from the Australian Research Council.

* To whom all correspondence should be addressed: Department of Biochemistry, University of Sydney, Sydney NSW 2006, Australia. Telephone: 61-2-9351-6031. Fax: 61-2-9351-4726. E-mail: r.christopherson@biochem.usyd.edu.au.

[1] Abbreviations: CA-asp, *N*-carbamyl-L-aspartate; CAD, trifunctional protein containing carbamyl phosphate synthetase (CPSase, EC 2.7.2.9), aspartate transcarbamylase (ATCase, EC 2.1.3.2), and dihydroorotase (EC 3.5.2.3); DHO, L-dihydroorotate; DHOase, dihydroorotase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HDDP, 2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylate; MES, 2-(*N*-morpholino)ethanesulfonic acid; rDHOase, recombinant dihydroorotase; TCEP•HCl, tris(2-carboxyethyl)phosphine hydrochloride.

[2] Amino acid residues of DHOase were numbered according to the published sequence (*1*).

10.1021/bi990859x CCC: $18.00
Published on Web 07/14/1999

glutamine and glutamate residues, respectively, contribute the carbonyl of the triad (*12*, *13*).

We report here about the substitution of the zinc atom at the active site of the hamster DHOase domain with several divalent metal ions and detailed kinetic analyses of the resultant metal−enzyme derivatives which provide information about the role of the bound Zn(II) in catalysis. These derivatives of DHOase should enable further study of the enzyme by spectroscopic techniques such as electronic absorption, circular dichroism, nuclear magnetic resonance, and electron spin resonance (*14*, *15*).

EXPERIMENTAL PROCEDURES

Materials and Methods. All the water that was used was purified by reverse osmosis followed by passage through a Millipore Reagent Water System (Millipore Co., Bedford, MA) to give a resistance of 18 MΩ. Ethylenediaminetetraacetic acid (EDTA), 8-hydroxyquinoline-5-sulfonic acid, 2-(*N*-morpholino)ethanesulfonic acid (MES), 1,10-phenanthroline, and pyridine-2,6-dicarboxylic acid were purchased from Sigma Chemical Co. (St. Louis, MO). L-[2-^{14}C]DHO and 2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylic acid (HDDP) were synthesized as described previously (*16*). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was from Pierce (Rockford, IL). All divalent metal ions that were used were the highest-reagent grade chloride salts from Sigma. To reduce zinc contamination, glassware and plasticware were soaked in 1 M HCl and diluted Extran (a biodegradable phosphate-free, alkaline cleaning solution; Merck Pty Ltd., Victoria, Australia), respectively, for at least 3 h, and then rinsed thoroughly with water. Metal standards for atomic absorption spectroscopy were purchased from BDH Ltd. (Poole, U.K.).

Atomic Absorption Spectrophotometry. Zinc, cobalt, manganese, cadmium, magnesium, calcium, nickel, and copper were identified on a SpectrAA·400 plus graphite furnace atomic absorption spectrophotometer (Varian, Palo Alto, CA). Five dilutions were made for each sample and standard, and duplicate samples of 20 μL were analyzed. Samples were diluted with blank solution [water for zinc and 0.1% (v/v) HNO_3 for other metals] so they would fall within the ranges of the standard curves, which were as follows: zinc, 0.01−1.5 ppb; cobalt, 5−50 ppb; manganese, 0.5−6 ppb; cadmium, 0.05−2 ppb; magnesium, 0.2−2 ppb; calcium, 1−6 ppb; nickel, 5−50 ppb; and copper, 5−50 ppb. Metal content was determined by linear regression to data from the five dilutions of the standard and unknown.

Protein Assay. Protein concentrations were determined by the Bradford assay using bovine serum albumin as the standard (*17*). Alternatively, protein was quantified by absorbance at 280 nm using a molar extinction coefficient of 29 800 M^{-1} cm^{-1} determined from the dry weight of pure DHOase.

Preparation of Recombinant Dihydroorotase (rDHOase). *E. coli* K strain SØ1263/pyrC$^-$, transformed with the plasmid, pCW25, containing the hamster DHOase domain with an extra 33 amino acid residues in the bridge region, was overexpressed and purified as described previously (*18*). The pure protein was stored in 20 mM Na·Hepes (pH 7.2), 10% (v/v) glycerol, 0.1 mM EDTA, and 1 mM DTT at −20 °C.

Preparation of Apo-DHOase and Metal Derivatives of DHOase. The apoenzyme was prepared by dialyzing pure rDHOase (1−4 mg of protein/mL; 5−12 mL) against chelating buffer (250 mL) containing 50 mM Na·MES, 25 mM pyridine-2,6-dicarboxylate, 1 mM EDTA, 30% (v/v) glycerol, 50 mM Na_2SO_4, and 0.5 mM DTT at pH 6.0 for 3 days with two changes of fresh chelating buffer (250 mL) at 24 and 48 h. The apo-rDHOase was concentrated to less than 3 mL using a Diaflo ultrafiltration cell and desalted by passage over a column of Sephadex G-25 (1.6 cm × 45 cm) eluted with column buffer [20 mM Na·MES (pH 6.3), 20% (v/v) glycerol, and 0.1 mM TCEP]. The apoenzyme eluted in the void volume was pooled and concentrated to approximately 4.0 mg of protein/mL.

Zn(II), Co(II), Mn(II), Cd(II), Mg(II), Ca(II), Ni(II), and Cu(II) derivatives of rDHOase were prepared by diluting the apoenzyme (4.0 mg of protein/mL) 4-fold with 50 mM Na·Hepes (pH 7.4), 30% (v/v) glycerol, and 0.1 mM TCEP, with a final divalent metal chloride concentration of 100 μM Zn(II), 500 μM Co(II), 1 mM Mn(II), 100 μM Cd(II), 1 mM Mg(II), 1 mM Ca(II), 1 mM Ni(II), or 100 μM Cu(II). The solutions were left at 4 °C, and the recoveries of enzymic activities of the respective metal derivatives of DHOase were followed. When the activity was maximal and constant, excess metal chloride was removed by Sephadex G-25 chromatography. The kinetic constants, metal content, and protein concentration of each metal derivative of DHOase were determined.

Enzyme Kinetics. DHOase was assayed in the degradative direction (DHO → CA-asp) as described previously (*16*). Reaction mixtures (23 μL) containing 50 mM Na·Hepes (pH 7.4) and 5% (v/v) glycerol with varying concentrations of L-[2-^{14}C]DHO (2−200 μM, 54 Ci/mol) were preincubated at 37 °C for 5 min. Reactions were initiated by adding pure DHOase (2 μL, 10 ng of protein), and aliquots (7 μL) were spotted onto poly(ethyleneimine)-cellulose chromatograms at 5 min intervals. Reaction velocities were determined by linear regression to these three time points, and K_m and V_{max} values were calculated by nonlinear regression to the Michaelis−Menten equation using Sigma-Plot (Jandel Scientific, Corte Madera, CA).

pH Studies and Data Analysis. The initial velocities of metal derivatives of DHOase over the pH range of 5.5−9.0 were determined. The reaction mixtures were buffered with Na·MES (pH 5.5−7), Na·Hepes (pH 7−8), or Tris-HCl (pH 8−9), and the concentration of DHO was maintained constant at 100 μM, well above the apparent K_m value (*5*). The reaction was initiated with 2 μL of enzyme (10 ng of protein), and the mixture was incubated at 37 °C for 5 min and then analyzed by thin-layer chromatography. Reaction velocities obtained at each pH value were fitted to eq 2 (*19*) using Sigma-Plot, where $(V_{max})_H$ is the maximal degradative reaction rate at a particular pH, V_{max} is the maximal rate when all the DHOase sites are in the appropriate ionic form, and K_a is the acid dissociation constant for a catalytic residue at the active site.

$$(V_{max})_H = \frac{V_{max}K_a}{K_a + [H^+]} \quad (2)$$

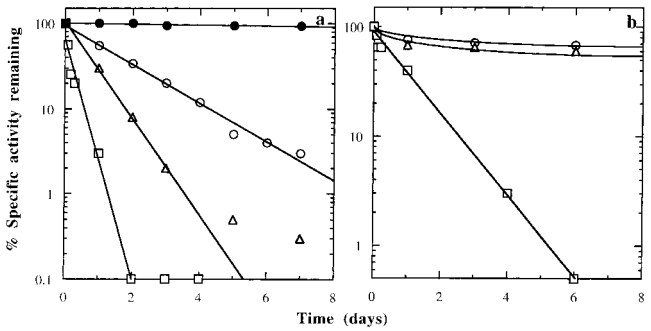

FIGURE 1: Inactivation of DHOase in the presence of the chelators, (a) pyridine-2,6-dicarboxylate and (b) EDTA, at various pH values. rDHOase (4.0 mg of protein/mL) was dialyzed at 4 °C against chelating buffer containing 25 mM pyridine-2,6-dicarboxylate or 25 mM EDTA, 50 mM Na_2SO_4, 30% (v/v) glycerol, 0.5 mM DTT, and 50 mM Na·MES or Na·Hepes: (●) control, at pH 7.3 in the absence of chelators, (□) pH 6.0, (△) pH 6.5, or (○) pH 7.3. DHOase activity was measured at the indicated times. The half-lives for the loss of DHOase activity in the presence of 25 mM pyridine-2,6-dicarboxylate were 2.5, 13, and 29 h at pH 6.0, 6.6, and 7.3, respectively.

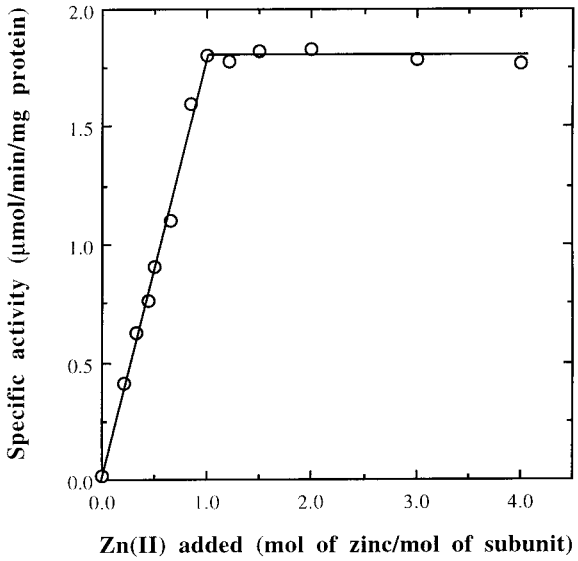

FIGURE 2: Relationship between specific enzymic activity and molar ratio of zinc to subunit of hamster DHOase. Standardized concentrations of $ZnCl_2$ and the apoenzyme were mixed as described in Experimental Procedures and incubated at 4 °C for 3 days. The DHOase activity was then determined.

RESULTS

Preparation of the Apoenzyme. Chelators such as 1,10-phenanthroline, EDTA, and pyridine-2,6-dicarboxylate have been used for removal of zinc from the active sites of the DHOases from *C. oroticum* and *E. coli* (*8*−*10*). In the study presented here, these chelators were tested for their ability to remove zinc from the active site of the recombinant hamster DHOase domain. Figure 1a shows that dialysis of rDHOase against pyridine-2,6-dicarboxylate causes time-dependent loss of activity, promoted by lowering the pH. The decay of activity with time is coordinated with the loss of zinc from the active site; Figure 2 shows that DHOase activity is zinc-dependent and maximal when one zinc atom is bound per enzyme subunit. EDTA removed the zinc at pH 6.0, but the extraction time was too long and ineffective at high pH (Figure 1b). 1,10-Phenanthroline also slowly removed zinc at pH 6.0, but 8-hydroxyquinoline and 8-hydroxyquinoline-5-sulfonate were unable to extract the zinc fully from the active site even at low pH (data not shown). Protein precipitation was rapid at low pH close to the isoelectric point of 5.7 (*18*). We found that inclusion of 30% (v/v) glycerol and 50 mM Na_2SO_4 in the chelating buffer kept the protein soluble and stable. The circular dichroism spectrum shows that wild-type rDHOase consists of a mixture of α-helix (36%) and β-sheet (41%), similar to the content of the DHOase fragment isolated after the elastase digestion of CAD (*20*). Removal of zinc from the active site had little effect on the CD spectrum (data not shown), suggesting that zinc is not required for the gross conformation of the enzyme.

Table 1: Kinetic Parameters[a] for the Degradative Activity and Metal Content of DHOase Derivatives

metal derivative	$(V_{max})_H$ (μmol min^{-1} mg^{-1})[b]	K_m (μM)	metal[d] content (mol of metal/ mol of subunit)	displaced by zinc[e]
native	2.15 ± 0.08	7.2 ± 0.7	1.02 ± 0.03 (Zn)	−
apo-rDHOase	*c*	−	0.01 ± 0.03 (Zn)	−
Zn−rDHOase	1.81 ± 0.07	6.8 ± 0.9	0.96 ± 0.07 (Zn)	−
Co−rDHOase	2.57 ± 0.07	3.6 ± 0.5	1.05 ± 0.04 (Co)	−
Mn−rDHOase	0.52 ± 0.02	5.3 ± 1.1	0.98 ± 0.02 (Mn)	yes
Cd−rDHOase	0.25 ± 0.02	9.1 ± 2.6	0.98 ± 0.11 (Cd)	yes
Ni−rDHOase	inactive	−	1.01 ± 0.02 (Ni)	yes
Cu−rDHOase	inactive	−	0.98 ± 0.04 (Cu)	yes

[a] The kinetic parameters were determined at pH 7.4 as described in Experimental Procedures. [b] $(V_{max})_H$ is the maximal activity at a particular pH. [c] Apo-rDHOase was prepared as described in Experimental Procedures and had less than 0.1% of the wild-type activity. [d] The metal ion analyzed is indicated in parentheses. [e] These metal derivatives were incubated with zinc and the enzymic activities increased.

Reconstitution of Apo-rDHOase with Other Divalent Metal Ions. Atomic absorption analysis shows that the recombinant hamster DHOase domain contains 1.0 atom of zinc per subunit, and dialysis of rDHOase against pyridine-2,6-dicarboxylate yielded the apoenzyme containing 0.01 atom of zinc per subunit with a specific activity of less than 0.1% of that of the original rDHOase (Table 1). Figure 3 demonstrates that upon addition of Zn^{2+}, Co^{2+}, Mn^{2+}, or Cd^{2+} ions to the apoenzyme, the enzymic activity increases to 130, 100, 30, or 14%, respectively, of the original activity after 3 days, but prolonged incubation with excess divalent metal ions caused some inhibition. The concentrations of metal ions added to the apoenzyme (Experimental Procedures) were carefully chosen as high concentrations of Zn^{2+}, Co^{2+}, Cd^{2+}, or Cu^{2+} ions can cause immediate protein precipitation.

Enzyme Kinetics and Metal Content. The kinetic parameters of the metal derivatives of DHOase were measured in the degradative direction at pH 7.4. Table 1 shows that all metal derivatives prepared contained approximately one atom of metal per enzyme subunit, and the V_{max} values were in the following order: Co(II)− → Zn(II)− → Mn(II)− → Cd(II)−rDHOase; there was no measurable activity for Ni(II)− and Cu(II)−rDHOase. Mg(II) and Ca(II) were also unable to restore enzymic activity, and atomic absorption spectroscopy showed that these two metal ions were not bound to the enzyme. The lower V_{max} value observed for the reconstituted Zn(II)−rDHOase compared with the original rDHOase is probably due to irreversible protein denaturation during dialysis at 4 °C for 2 weeks. Mn(II)−rDHOase has a slightly lower K_m value for DHO than that of the wild-type enzyme; Cd(II)−rDHOase has a slightly

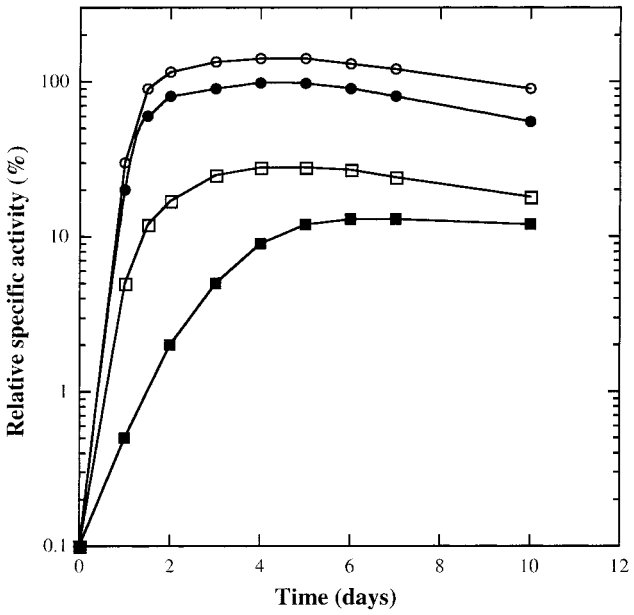

FIGURE 3: Reconstitution of apo-rDHOase with (○) Co(II), (●) Zn(II), (□) Mn(II), and (■) Cd(II). The apoenzyme (1.0 mg of protein/mL) was incubated in 50 mM Na·Hepes (pH 7.4), 30% (v/v) glycerol, 0.1 mM TCEP with 500 μM $CoCl_2$, 100 μM $ZnCl_2$, 1 mM $MnCl_2$, or 100 μM $CdCl_2$. Enzymic activity was followed as a function of time with 100% activity being equivalent to that of the original native DHOase. Incubation with excess divalent metal chloride after maximal activity was attained resulted in inhibition of the enzyme.

higher K_m, and Co(II)−rDHOase has a 2-fold lower K_m (Table 1). We also observed an increase in enzymic activity for Mn(II)−, Cd(II)−, Ni(II)−, and Cu(II)−rDHOase upon addition of zinc, suggesting displacement of metal ion at the active site by zinc.

pH−Rate Experiments. The DHOase activity of mouse CAD in the biosynthetic and the degradative directions is pH-dependent, consistent with ionization of a catalytic residue at the active site with a pK_a value of 7.1 (*19*). Acidic pH favors the biosynthetic reaction (CA-asp → DHO), while alkaline pH favors the degradative reaction (DHO → CA-asp). We found a similar dependence of degradative activity on pH with Zn(II)−rDHOase (Figure 4a), and a pK_a value of 6.56 was derived from fitting the data to eq 2 (Table 2). To determine whether the metal ion at the active site influences this pH dependence, the degradative activities of the four active metal derivatives of DHOase were measured between pH 5.5 and 9.0. The variation of log(V) as a function of pH for Co(II)−, Mn(II)−, and Cd(II)−rDHOase reflects a similar dependence of activity upon pH (Figure 4b−d) with pK_a values similar to that of Zn−rDHOase (Table 2). In all instances, the pH−log(V) profiles reflect ionization of the catalytic residue with a pK_a in the range of 6.4−6.9.

Absorption Spectra. The absorption spectrum of Co(II)−rDHOase was recorded at pH 6.0 and 8.0. Figure 5 shows the d−d transitions for Co(II)−rDHOase from 400 to 700 nm. The Co(II) spectrum from 500 to 650 nm may be complicated by light scattering due to high protein concentrations (8.5 mg of protein/mL; *21*). To allow for such an effect, a difference spectrum relative to the Zn(II) enzyme was obtained (Figure 5; *22*, *23*). Four-coordinated (pseudo-tetrahedral) Co(II) complexes are characterized by absorption maxima at wavelengths above 600 nm, strongly structured

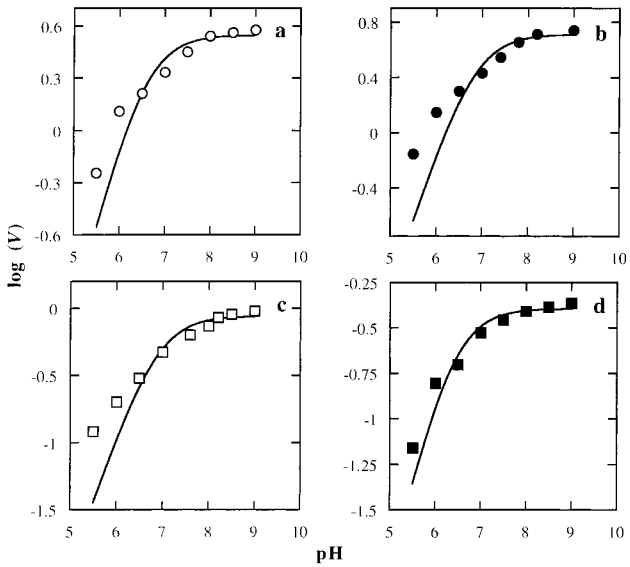

FIGURE 4: Variation of log(V) with pH for the degradative reaction catalyzed by metal derivatives of DHOase. The activities of (a) Zn(II)−rDHOase, (b) Co(II)−rDHOase, (c) Mn(II)−rDHOase, and (d) Cd(II)−rDHOase were determined at the indicated pH values as described in Experimental Procedures. V is the turnover number of the enzyme (moles of substrate converted per mole of active site per second). The curves were generated using eq 2 with the fitted parameters from Table 2.

Table 2: pK_a and V_{max} Values for the Degradative Activity of Metal Derivatives of DHOase[a]

metal derivative	pK_a	V_{max} (μmol^{-1} min^{-1} mg^{-1})
Zn−rDHOase	6.56 ± 0.12	3.52 ± 0.2
Co−rDHOase	6.83 ± 0.17	5.16 ± 0.33
Mn−rDHOase	6.87 ± 0.12	0.87 ± 0.04
Cd−rDHOase	6.41 ± 0.08	0.40 ± 0.02

[a] The pK_a and V_{max} values were obtained by fitting DHOase activities at each pH value to eq 2 as described in Experimental Procedures. V_{max} denotes the maximal rate when all DHOase sites are in the basic ionic form.

peaks, and molar extinction coefficients of greater than 300 M^{-1} cm^{-1} (*24*). Six-coordinated (pseudo-octahedral) Co(II) complexes are characterized by maxima at wavelengths below 600 nm, little structure in the peaks, and molar extinction coefficients of less than 50 M^{-1} cm^{-1} (*24*). Five-coordinated (any geometry) Co(II) complexes typically have molar extinction coefficients between 50 and 200 M^{-1} cm^{-1} (*24*). At pH 8.0, there are two distinct maxima at 520 and 570 nm with molar extinction coefficients of 195 and 210 M^{-1} cm^{-1}, respectively, and a minimum at 550 nm (ϵ = 180 M^{-1} cm^{-1}), consistent with pentacoordination. At pH 6.0, the maximum at 520 nm and minimum at 550 nm observed at pH 8.0 were absent, and the maximum at 570 nm was reduced to 60 M^{-1} cm^{-1}, consistent with pentacoordination. Addition of the DHOase inhibitor, 2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylate (HDDP, K_i = 0.74 μM; *16*), to Co(II)−rDHOase at pH 8.0 induced an immediate and marked change in the spectrum (Figure 5). The maximum at 520 nm was reduced to a shoulder, and the maximum at 570 nm had a molar extinction coefficient of 165 M^{-1} cm^{-1}, indicative of pentacoordination possibly due to coordination of the two oxygens of the carboxylate at C6 of HDDP to Co(II) at the active site (*12*).

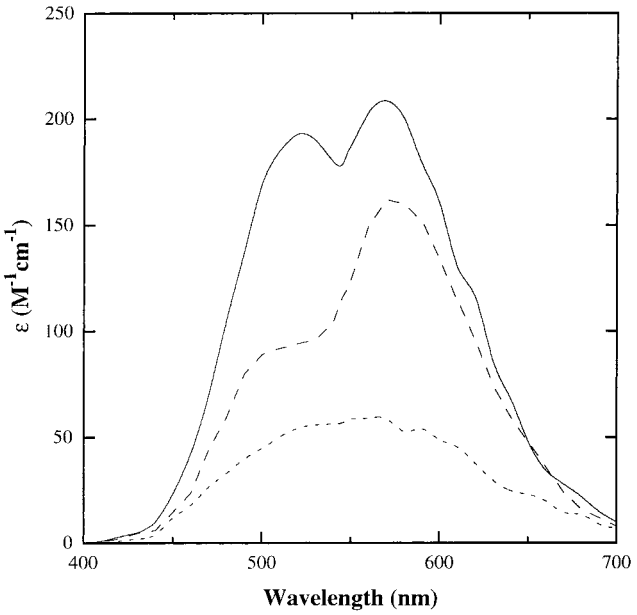

FIGURE 5: Visible absorption spectra of Co(II)−rDHOase at high pH (solid line) and low pH (dotted line), and in the presence of HDDP (dashed line). The spectra of Co(II)−rDHOase (8.5 mg of protein/mL) in 50 mM Tris-HCl (pH 8.0) and 50 mM Na·MES (pH 6.0) with 10% (v/v) glycerol and 0.1 mM TCEP were recorded from 400 to 700 nm on a Hewlett-Packard 8452A diode array spectrophotometer. A final concentration of 8 mM HDDP was added to Co(II)−rDHOase in Tris-HCl (pH 8.0), 10% (v/v) glycerol, and 0.1 mM TCEP to give a ratio of 40 molecules of HDDP per active site. The mixture was incubated at room temperature for 10 min, and the spectrum was recorded.

DISCUSSION

The active site of DHOase has a high affinity and specificity for Zn(II). Zinc has a direct catalytic role at the active site and is coordinated to three histidine ligands, two in close proximity (His15 and His17), and the third ligand is probably His158 (*12*). The active holoenzyme was formed when one atom of zinc was bound at the active site of rDHOase (Figure 2). The apoenzyme obtained using pyridine-2,6-dicarboxylate contained approximately 0.01 atom of zinc per subunit with 0.1% of the enzymic activity of the wild type. A mechanism for removal of zinc from carbonic anhydrase by pyridine-2,6-dicarboxylate has been proposed where the reaction proceeds through the formation of a ternary complex with the zinc at the active site, followed by slow dissociation of the zinc−chelator complex from the active site forming the apoenzyme (*25*, *26*). The structural similarity of pyridine-2,6-dicarboxylate to DHO suggests that this chelator may enter the active site readily and form a ternary complex with the bound zinc. Lowering the pH to 6.0 increases the rate of zinc extraction, possibly by protonating the histidine ligands at the active site and reducing the zinc affinity of DHOase. At pH 5.0, zinc may dissociate spontaneously from *E. coli* DHOase (*10*). Therefore, at pH 6.0, zinc ion may slowly dissociate from the active site of hamster DHOase, providing an explanation of how zinc removal by EDTA or 1,10-phenanthroline is slow and only occurs at low pH. Both chelators are bulky and may not enter the active site, but at low pH, zinc ions that slowly dissociate from the active site would be rapidly scavenged by these chelators. Zinc has been removed from *C. oroticum* DHOase, which contains two zinc atoms per subunit, using EDTA or 1,10-phenanthroline at pH 6.5 (*8*, *9*). The zinc atom at the active site of *E. coli* DHOase could only be removed completely using pyridine-2,6-dicarboxylate at pH 5.0 (*10*). These observations suggest that the active sites of the DHOases from these three species may have different affinities for zinc.

Reconstitution of apo-rDHOase with zinc, cobalt, manganese, or cadmium restored enzymic activity. The order of V_{max} values for the metal derivatives of DHOase was as follows: Co(II)− → Zn(II)− → Mn(II)− → Cd(II)−rDHOase. Co(II) has been used to replace Zn(II) in a number of zinc-enzyme complexes, and in many cases, full catalytic activity was restored (*27*). Zinc and cobalt have similar ionic radii and readily accept distorted geometries in model complexes with coordination numbers of four to six (*27*−*29*). Cobalt, being a d^7 metal ion, is an exceptional probe for studying the metal environment at the active site of DHOase. The electronic absorption spectra of Co(II)−rDHOase at high and low pH (Figure 5) are consistent with pentacoordinate geometry about the bound cobalt. This geometry could arise from coordination of the cobalt by the three histidine ligands and two water molecules. The crystal structure of Co(II)−thermolysin (EC 3.4.24) exhibited cobalt coordinated to His142, His146, Glu166, and two water molecules (*30*). It was proposed that the greater catalytic activity of Co(II)−thermolysin relative to the zinc-substituted enzyme may be due to enhanced stabilization of the transition state. The proposed enzyme−transition state complex for DHOase may also involve pentacoordinate geometry of the zinc atom (*12*); the Co(II)−rDHOase may more readily assume the 5-fold coordination geometry of the transition state, making it more effective in catalysis than Zn(II)−rDHOase. The Co(II) derivatives of DHOase from *C. oroticum* (*9*) and *E. coli* (*10*) are also more active than the native bacterial DHOase, and the bound cobalt ion is pentacoordinated in *E. coli* DHOase (*10*). Mn(II)- and Cd(II)-substituted Zn−enzyme complexes are generally less active or inactive, perhaps resulting from conformational changes that disrupt the arrangement of essential catalytic residues (*30*) or the inability of manganese and cadmium to adopt the pentacoordinate geometry required in the transition state of catalysis. The K_m values for DHO determined here for these metal derivatives (Table 1) are similar to the native enzyme, suggesting they have similar affinities for DHO. The Co(II)−DHOase from *E. coli* has a slightly lower K_m value for DHO compared to that of native DHOase (*10*), as observed here for the hamster DHOase domain (Table 1).

DHOase activity is pH-dependent (Figure 4), and the derived pK_a values for the metal derivatives varied little with metal substitution (6.4−6.9, Table 2). The pK_a of 6.56, derived from the pH−rate profile for Zn−rDHOase, could be attributed to ionization of an enzyme−metal aquo complex, [i.e., $Me(L_n)(H_2O) \leftrightarrow Me(L_n)(OH^-) + H^+$], a carboxyl or an imidazolyl group. The acidity of such a coordinated water molecule would be dependent on the metal involved (*31*, *32*). For example, the activity-linked pK_a for carbonic anhydrase shifts from 7.5 to 9.5 when Cd(II) is substituted for Zn(II) at the active site, suggesting that the pK_a is that of a metal-coordinated water molecule (*33*). Coordination of H_2O to the "harder" metal ion, Zn(II), would make the metal aquo complex a stronger Lewis acid than coordination to the "softer" metal ion, Cd(II) (*34*). The pK_a

values derived from the pH−rate profile for Zn(II)− and Cd(II)−rDHOases here are similar (Figure 4), strongly suggesting that the derived pK_a value of the enzyme−substrate complex does not represent an enzyme−metal aquo complex. A pK_a of 6.56 would be high for a carboxyl group unless it was placed in a hydrophobic environment (*24*, *31*). Asp230 was proposed to be located at the active site of DHOase and may act as a general acid or base in catalysis (*12*), but its environment is unknown. Inactivation of mouse DHOase by diethyl pyrocarbonate suggested at least one histidine residue involved in catalysis (*19*), and it is possible that the catalytic residue with a pK_a of 6.56 is a histidine. Site-directed mutagenesis and diethyl pyrocarbonate incorporation suggested that His234 participates in catalysis (*11*). At high pH, it could act as a general base deprotonating the zinc-bound water to hydroxyl ion which would then attack the C6 carbonyl of DHO. At low pH, this residue would be protonated and the positive charge could electrostatically stabilize the negatively charged substrate, CA-asp.

The absorption spectra of Co(II)−rDHOase (Figure 5) show that the environment of the bound cobalt at high pH is different from that at low pH, suggesting a change in the microenvironment at the active site which may be important for substrate (CA-asp or DHO) recognition and catalysis. Addition of the inhibitor, HDDP ($K_i = 0.74\ \mu$M; *16*), changes the spectrum, suggesting direct interaction with the cobalt ion, providing an explanation for the potency of this inhibitor.

Prolonged incubation of fully activated metal derivatives of DHOase in the presence of excess amounts of that metal ion has an inhibitory effect (Figure 3). However, upon removal of excess metal ions by Sephadex G-25 chromatography, the maximal activity was restored. Such inhibition has also been observed for other zinc−enzyme complexes, for example, thermolysin and adenosine deaminase (*30*, *35*). For thermolysin, in the presence of excess zinc, a second zinc ion interacts with His231 within 3.2 Å of the zinc of native thermolysin, blocking entry of the substrate into the active site (*30*). In the case of adenosine deaminase, Mn^{2+}, Cu^{2+}, and Zn^{2+} were found to be competitive inhibitors of the enzyme, while Cd^{2+} and Co^{2+} were noncompetitive inhibitors (*35*).

In conclusion, hamster DHOase requires a single bound zinc at the active site for catalytic activity. Divalent metal ions that have affinity for histidine can replace the zinc ion; those which have flexible coordination geometries can also catalyze the reaction. Substitution of cobalt, manganese, or cadmium for zinc at the active site of DHOase shows that the metal ion strongly influences the maximal activity of the enzyme but with little effect on the pK_a values derived from pH−rate proflies or on the K_m values for DHO. We conclude that a metal−enzyme aquo complex is not involved with the initial binding of substrate, but may be involved with the rate-determining step of catalysis.

ACKNOWLEDGMENT

We thank Dr. Neal K. Williams for construction of the *E. coli* K strain SØ1263/pyrC$^-$ transformed with the plasmid, pCW25, which overexpresses the recombinant hamster dihydroorotase domain.

REFERENCES

1. Williams, N. K., Simpson, R. J., Moritz, R. L., Yin, P., Crofts, L., Minasian, E., Leach, S. J., Wake, R. G., and Christopherson, R. I. (1990) *Gene 94*, 283−288.
2. Jones, M. E. (1980) *Annu. Rev. Biochem. 49*, 253−279.
3. Simmer, J. P., Kelly, R. E., Rinker, A. G., Jr., Zimmermann, B. H., Scully, J. L., Kim, H., and Evans, D. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 174−178.
4. Kelly, R. E., Mally, M. I., and Evans, D. R. (1986) *J. Biol. Chem. 261*, 6073−6083.
5. Christopherson, R. I., and Jones, M. E. (1980) *J. Biol. Chem. 255*, 3358−3370.
6. Taylor, W. H., Taylor, M. L., Balch, W. E., and Gilchrist, P. S. (1976) *J. Bacteriol. 127*, 863−873.
7. Washabaugh, M. W., and Collins, K. D. (1984) *J. Biol. Chem. 259*, 3293−3298.
8. Pettigrew, D. W., Bidigare, R. R., Mehta, B. J., Williams, M. I., and Sander, E. G. (1985) *Biochem. J. 230*, 101−108.
9. Pettigrew, D. W., Mehta, B. J., Bidigare, R. R., Choudhury, R. R., Scheffler, J. E., and Sander, E. G. (1985) *Arch. Biochem. Biophys. 243*, 447−453.
10. Brown, D. C., and Collins, K. D. (1991) *J. Biol. Chem. 266*, 1597−1604.
11. Zimmermann, B. H., Kemling, N. M., and Evans, D. R. (1995) *Biochemistry 34*, 7038−7046.
12. Williams, N. K., Manthey, M. K., Hambley, T. W., O'Donoghue, S. I., Keegan, M., Chapman, B. E., and Christopherson, R. I. (1995) *Biochemistry 34*, 11344−11352.
13. Christianson, D. W., and Alexander, R. S. (1990) *Nature 346*, 225.
14. Bertini, I., Luchinat, C., and Viezzoli, M. S. (1986) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, W., and Zeppezauer, M., Eds.) pp 27−47, Birkhäuser, Boston.
15. Coleman, J. E., and Gettins, P. (1986) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, W., and Zeppezauer, M., Eds.) pp 77−99, Birkhäuser, Boston.
16. Christopherson, R. I., Schmalzl, K. J., Szabados, E., Goodridge, R. J., Harsanyi, M. C., Sant, M. E., Algar, E. M., Anderson, J. E., Armstrong, A., Sharma, S. C., Bubb, W. A., and Lyons, S. D. (1989) *Biochemistry 28*, 463−470.
17. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
18. Williams, N. K., Peide, Y., Seymour, K. K., Ralston, G. B., and Christopherson, R. I. (1993) *Protein Eng. 6*, 333−340.
19. Christopherson, R. I., and Jones, M. E. (1979) *J. Biol. Chem. 254*, 12506−12512.
20. Hemmens, B., and Carrey, E. A. (1995) *Eur. J. Biochem. 231*, 220−225.
21. Shriver, D. F., Atkins, P. W., and Langford, C. H. (1990) *Inorganic Chemistry*, pp 493, W. H. Freeman and Co., New York.
22. Giedroc, D. P., Keating, K. M., Williams, K. R., Konigsberg, W. H., and Coleman, J. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8452−8456.
23. Pan, T., and Coleman, J. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3145−3149.
24. Bertini, I. (1983) in *The Coordination Chemistry of Metalloenzymes* (Bertini, I., Drago, R. S., and Luchinat, C., Eds.) pp 1−18, D. Reidel Publishing Co., Boston.
25. Kidani, Y., and Hirose, J. (1977) *J. Biochem. 81*, 1383−1391.
26. Kidani, Y., and Hirose, J. (1986) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, W., and Zeppezauer, M., Eds.) pp 59−75, Birkhäuser, Boston.
27. Vallee, B. L., and Williams, R. J. P. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 498−505.
28. Vallee, B. L., and Glades, A. (1984) *Adv. Enzymol. 56*, 283−430.
29. Lipscomb, W. N., and Sträter, N. (1996) *Chem. Rev. 96*, 2375−2433.
30. Holland, D. R., Hausrath, A. C., Juers, D., and Matthews, B. W. (1995) *Protein Sci. 4*, 1955−1965.
31. Auld, D. S., and Vallee, B. L. (1970) *Biochemistry 9*, 4352−4359.
32. Makinen, M. W., Kuo, L. C., Dymowski, J. J., and Jaffer, S. (1979) *J. Biol. Chem. 254*, 356−366.

33. Bauer, R., Limkilde, P., and Johansen, J. T. (1976) *Biochemistry 15*, 334−342.
34. Coleman, J. E. (1986) in *Zinc Enzymes* (Bertini, I., Luchinat, C., Maret, W., and Zeppezauer, M., Eds.) pp 49−58, Birkhäuser, Boston.
35. Cooper, B., Sideraki, V., Wilson, D. K., Dominguez, D. Y., Clark, S. W., Quiocho, F. A., and Rudolph, F. B. (1997) *Protein Sci. 6*, 1031−1037.

BI990859X