Function of Conserved Histidine Residues in Mammalian Dihydroorotase†

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ABSTRACT: Dihydroorotase (DHOase, EC 3.5.2.3) catalyzes the reversible cyclization of carbamyl aspartate to form dihydroorotate, the third step in *de novo* pyrimidine biosynthesis. In mammals this activity is carried by the zinc-containing domain of the 243 kDa multifunctional protein CAD. We have replaced conserved residues in the cloned 46 kDa DHOase domain by site-directed mutagenesis. Mutants His1471Ala and His1473Ala lacked catalytic activity, judging by their failure to complement a DHOase-deficient *Escherichia coli* strain, and were unable to coordinate the active site zinc ion in zinc blotting experiments. This result confirmed earlier predictions. A mutant protein in which the third suspected zinc ligand was changed, Glu1512Asn, had a k_{cat} similar to that of the intact CAD molecule and a K_m similar to that of the wild-type recombinant DHOase, observations that argue against a role for glutamate 1512 in catalysis. Mutant His1590Asn had no measurable catalytic activity. This histidine residue was tentatively identified as the third zinc ligand by the failure of the mutant to bind the full complement of zinc in atomic absorption measurements. Mutant His1690Asn had a k_{cat} 34-fold lower and a K_m 9-fold higher than those of wild-type recombinant. The kinetic parameters of the mutant His1642Asn were also altered, but to a lesser extent. Diethyl pyrocarbonate (DEPC) was shown previously to inactivate mammalian DHOase. Spectroscopic studies and [^{14}C]DEPC incorporation demonstrated that the loss of activity is associated with the modification of approximately two histidine residues located at or near the active site. Atomic absorption showed that reaction with DEPC did not result in expulsion of the zinc ion, suggesting that histidine residues 1471, 1473, and 1590 do not react with DEPC. Moreover, incorporation of [^{14}C]DEPC into mutants His1590Asn and His1642Asn was similar to that observed for the wild-type protein. In contrast, the incorporation of DEPC into the mutant His1690Asn was reduced, and substrate no longer affected the reaction. Furthermore, the strong pH dependence of the catalytic reaction was significantly decreased in this mutant. Our results suggest that the active site zinc is coordinated by three histidines, His1471, His1473, and His1590, and that His1690 is located at the active site and participates in catalysis.

Dihydroorotase (DHOase)[1] (L-5,6-dihydroorotase amidohydrolase, EC 3.5.2.3) catalyzes the reversible cyclization of carbamyl aspartate to form dihydroorotate, the third step in *de novo* pyrimidine biosynthesis. In mammals, the activity is carried by the multifunctional protein CAD, which also carries the activities of the first two steps in the pathway, carbamyl phosphate synthetase (CPSase) and aspartate transcarbamylase (ATCase). The hamster CAD molecule is a hexamer consisting of six identical polypeptides, each having a molecular mass of 242 kDa. Limited proteolysis and sequence analysis have shown that the DHOase activity is associated with a discrete domain of the protein (Kelly et al., 1986; Simmer et al., 1990). A 44 kDa fragment containing the DHOase domain has been purified from controlled elastase digests of hamster CAD (Kelly et al., 1986). The isolated domain exhibits kinetic behavior similar to that of the intact protein and exists in solution as a dimer of ~88 kDa. Each monomer contains a tightly bound zinc that may be involved in catalysis (Christopherson & Jones, 1979, 1980; Kelly et al., 1986). Monofunctional dihydroorotases have been isolated from *Escherichia coli* (Washabaugh & Collins, 1984) and *Clostridium oroticum* (Pettigrew et al., 1985) and are also zinc-containing dimers.

Alignment of the DHOase coding region of CAD with other available DHOase sequences shows three highly conserved regions that are likely to contain active site residues and zinc ligands (Simmer et al., 1990). Recently, we and others reported the cloning and overexpression of sequences corresponding to the CAD DHOase domain in *E. coli* (Zimmermann & Evans, 1993; Williams et al., 1993). The kinetic parameters of the purified recombinant proteins are similar to those of the proteolytic DHOase domain and the intact protein (Zimmermann & Evans, 1993; Williams et al., 1993). Here we report our attempts to determine the roles of different conserved residues in catalysis and zinc binding by site-directed mutagenesis, chemical modification, and zinc atomic absorption.

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[1] Abbreviations: CAD, multifunctional protein that catalyzes the first three steps in pyrimidine biosynthesis in higher eukaryotes; DEPC, diethyl pyrocarbonate; DHOase, dihydroorotase (EC 3.5.2.3); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; kDa, kilodaltons; MES, 2-morpholinoethanolsulfonic acid; PAR, 4-(2-pyridylazo)resorcinol; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes, agarose, and acrylamide were obtained from Bethesda Research Laboratories, and casamino acids were from Difco Laboratories. Electrophoresis standards, unlabeled diethyl pyrocarbonate, and [*carbonyl*-^{14}C]diethyl pyrocarbonate, specific activity 2.1 mCi/mmol, were obtained from Sigma. Oligonucleotides used for sequencing and site-directed mutagenesis were synthesized by the Wayne State Macromolecule Core Facility.

Cell Strains and Vectors. E. coli strain EK1104 and pEK81, the pEK2-derived expression vector, were the gifts of Dr. E. R. Kantrowitz (Nowlan & Kantrowitz, 1985). The dihydroorotase-deficient *E. coli* cell strain, X7014, cgsc 5358 (Semple & Silbert, 1975), was obtained from the Yale *E. coli* Genetic Stock Center. The minimal medium was the same as that used in Zimmermann and Evans (1993), except that the ampicillin concentration was raised to 200 μg/mL for EK1104. Mutant transformed EK1104 was harvested 27 h after inoculation of 2 L minimal medium containing 10 μg/mL uracil. *E. coli* strains MV1190 and CJ236 and helper phage M13K07 used for site-directed mutagenesis were obtained from Bio-Rad.

Construction of Mutants. Site-directed mutations were constructed by the method of Kunkel (1985), using a Muta-Gene Phagemid In Vitro Mutagenesis Kit, Version 2, from Bio-Rad, pBZ22, a plasmid encoding the recombinant CAD dihydroorotase domain (Zimmermann & Evans, 1993), and the following oligonucleotides: 5′-AAGGTGCACA-GCGACGTCGATC-3′, H1471A; 5′-GAAGGTGCAC-GTTAACGTCGATC-3′, H1471N; 5′-CTCCCGAAGG-GCCACATGGACG-3′, H1473A; 5′-GCAGGGGCATTA-ATGATGGGG-3′; D1512N; 5′-CGCTCTGCATTGGC-CACAATG-3′, H1590N; 5′-AGGAAGAGGTTGTGGGG-TGCG-3′, H1642N; 3′-TCCAGGTATTGGGAGCGTG-G-5′, H1690N (nucleotides differing from the wild-type sequences are underlined). Oligonucleotides for three of the mutants were designed to add (D1512N, *Ase*I; H1471N, *Hpa*I) or destroy (H1473A, *Apa*L1) restriction sites, and the presence of the mutations was confirmed by restriction analysis. Mutants H1590N, H1642N, and H1690N were confirmed by nucleotide sequencing.

Nucleotide Sequencing. Mutants were sequenced by the Sanger dideoxy method (Sanger et al., 1977) using a Sequenase Version 2 DNA Sequencing Kit from United States Biochemical.

Protein Purification. Wild-type and mutant recombinant proteins were purified according to Zimmermann and Evans (1993), with minor changes. Washed cells could be stored as pellets at −70 °C with no loss in yield. To maximize the yield, 150 mM benzamidine was included in the sonication buffer, and the time between the sonication of the cells and the start of dialysis was limited to 3 h or less. The total volume of the DEAE-Sephacel gradient was increased to 100 mL. Mutant protein-containing fractions were identified by Western blotting with polyclonal antibodies directed against CAD DHOase (Kelly et al., 1986).

The CAD proteolytic fragment carrying DHOase activity was prepared according to Kelly and co-workers (1986).

Electrophoresis and Electroblotting. SDS−polyacrylamide gel electrophoresis was carried out on 1.5 mm, 7.5−15% gradient slab gels with 5% stacking gels using the buffer system described by Laemmli (1970). Gels were run using a Hoeffer Model SE650 gel apparatus and stained with Coomassie Brilliant Blue R.

Protein was transferred from SDS−polyacrylamide gels to nitrocellulose (Schleicher & Schuell) or Imobilon-P (Millipore) membranes using a Hoeffer Model TE42 Transphor for a minimum of 3 h at 6 V/cm. Processing of the nitrocellulose for Western blots and of Imobilon-P for amino acid sequencing was described previously (Zimmermann & Evans, 1993).

Amino Acid Sequencing. Amino acid microsequence analysis was obtained by automated Edman chemistry on an Applied Biosystems gas phase sequenator (Model 470) with on-line HPLC (Model 120) and a Nelson analytical chromatography data system in the Wayne State Macromolecular Core Facility.

Protein Assays. Protein was measured by the method of Bradford (1976) with reagents from Bio-Rad, using bovine serum albumin as a standard.

Enzyme Kinetics and pH Studies. Data for dihydroorotate saturation curves of the mutants were obtained using the colorimetric assay (Prescott & Jones, 1969). Assay mixtures containing 0.5 mL of 100 mM Tris−acetate (pH 8.3) with varying concentrations of dihydro-L-orotate were preincubated at 37 °C for 10 min. Reactions were initiated by adding 3.5 μL of pBZ22 recombinant protein or 50 μL of mutant proteins, incubated at 37 °C for 20 (wild type) or 60 min (mutants), and then quenched. For the pH studies, the assay mixtures were buffered with 100 mM HEPES (pH 7.0, 7.5, 8.0) or 100 mM MES (pH 6.5, 7.0). Kinetic parameters were obtained by a nonlinear least-squares fit to the Michaelis−Menten equation using the program MINSQ (Micromath).

Diethyl Pyrocarbonate Inactivation. Stock solutions of 50−250 mM DEPC were prepared in absolute ethanol and stored at −20 °C. Solutions of unlabeled DEPC were stable for several months, while radiolabeled DEPC decayed over time and was used within 3 weeks of opening an ampule. Stock solutions were quantified by adding aliquots to 20 mM MES and 10 mM *N*-acetylhistidine (pH 6.0) and calculating the resulting N^{α}-ethoxycarbonylhistidine derivative from the absorbance at 242 nm by using a difference extinction coefficient of 3900 M^{-1} cm^{-1} (Choong et al., 1977). A range of difference extinction coefficients is reported in the literature (Miles, 1977). We used the value reported by Choong and co-workers because it appeared to most accurately reflect the concentration of DEPC in stock bottles (6.9 M) upon back calculation following absorption measurements with solutions of *N*-acetylhistidine. The concentration of Zn^{2+}−PAR_2 complex was calculated by using $\Delta\epsilon_{500nm} = 6.6 \times 10^4$ M^{-1} cm^{-1} (Hunt et al., 1985). All spectrophotometric measurements were made on a Perkin-Elmer Lambda 5 UV/vis spectrophotometer with cuvette holders thermostated at 24 °C.

DEPC radiolabeling experiments were carried out as described in the following. A final concentration of 5 mM [*carbonyl*-^{14}C]DEPC was added to a protein solution containing mutant or wild-type recombinant protein at 0.04−0.10 mg/mL and 180 mM HEPES (pH 7.0), in the presence or absence of 5 mM dihydroorotate. Aliquots from the incubation mixtures containing the wild-type enzyme were withdrawn during the incubation to monitor loss of activity using the colorimetric assay. After 1 h of incubation at room temperature with DEPC, the unreacted modification reagent

was quenched by adding *N*-acetylhistidine to a concentration of 28 mM. After an additional 30 min at room temperature, the mixture was placed on ice, and a carrier protein, lysozyme, was added to a concentration of 0.25 mg/mL. Two methods, dialysis and acetone precipitation, were used to separate the labeled protein from N^{α}-ethoxycarbonylhistidine and unreacted DEPC. In the dialysis method, the protein solution was transferred to dialysis tubing (Spectrum Medical Industries, Spectropor, molecular weight cut-off 12000−14000) and placed in a screw-top bottle in the cold room with 500 μL of protein solution per 200 mL of dialysis buffer (50 mM HEPES (pH 7.4), 1.0 mM DTT, 5% glycerol, and 150 mM NaCl). The first two dialysis buffer solutions included 1 mM *N*-acetylhistidine. Dialysis buffer was changed every 45 min, and the course of dialysis was monitored by counting 0.5 mL of the buffer. Seven to eight buffer changes, including one overnight step (usually the fifth buffer change), were necessary to reduce the radioactivity of the buffer to background levels. After the dialyzed solution was removed from the tubing, a portion was used to measure protein concentration with a Bradford assay. Four volumes of Protosol (Dupont) were added to the remaining solution, which was heated at 55 °C for 30 min and then counted. The Bradford solution was also counted. We observed little difference between the number of counts recovered from Protosol-containing heat-treated samples and Bradford solutions. Precedent exists for retention of the carbethoxy modification of histidine during heat treatment (Li et al., 1993; Ko et al., 1991). The expected breakdown product of a modified histidine residue is $^{14}CO_2$ (Li et al., 1993). Product literature from Dupont indicates that Protosol is a superior absorber of CO_2 and can be used to measure CO_2 produced by histidine decarboxylase.

In the acetone precipitation method, 10 vol of ice cold acetone were added to the labeled protein solution. After 10 min on ice, the protein was precipitated by centrifuging for 5 min at 14 000 rpm in the cold room. The supernatant was removed, and the pellet was resuspended in 100 μL of buffer (50 mM HEPES (pH 7.4), 1.0 mM DTT, 5% glycerol, and 150 mM NaCl). The first two resuspension buffer solutions included 1 mM *N*-acetylhistidine. Failure to resuspend protein pellets resulted in protein solutions with anomalously high counts. The precipitation and resuspension steps were repeated until the supernatant reached background levels (7−8 times). After the final resuspension, a portion of the solution was used to measure protein with the Bradford assay. Both the Bradford solution and the remaining solution were counted. Dialysis and acetone precipitation yielded similar values for counts incorporated.

Atomic Absorption Measurements. Water was glass-distilled and passed through a primary deionization unit and a 0.2 μM postfilter cartridge (Millipore Corp.), with final filtration through an Ultrapure mixed-bed resin column (Barnstead Co.). Water prepared in this manner contains 0.02 ng of Zn/mL, is considered zinc-free, and was used for acid cleaning and to make all solutions. Plastic containers used for dialysis, pipet tips, and Eppendorf tubes were acid-cleaned first by rinsing with 1:40 nitric acid/water (vol/vol) and then by rinsing three times with zinc-free water. No glassware was used during sample preparation. Spectra/Por dialysis tubing (Spectrum Medical Industries) was made metal-free (Auld, 1988) by heating at 75 °C for 2 h in zinc-free water, with intermittent stirring using an acid-cleaned plastic rod, in an acid-cleaned plastic beaker. The water was decanted and the procedure was repeated three more times. Samples were concentrated under vacuum using a Savant SpeedVac system. Approximately 0.3 mL of each sample was dialyzed for 15−17 h at pH 8.0 against (1) 10 mM Tris Cl, 10 mM EDTA; (2) 10 mM Tris Cl, 10 mM EDTA; (3) 10 mM Tris Cl; and (4) 10 mM Tris Cl. The last two buffers contained a dialysis bag filled with 1 mL of Chelex 100 (Bio-Rad) prepared according to Martin (1988). The sample volume:dialysis buffer volume ratio was 1 mL:2 L. Latex gloves were acid-cleaned prior to handling dialysis tubing. Protein concentrations were measured after dialysis. Dialyzed samples were diluted 1:1 with Ultrex II concentrated nitric acid (J. T. Baker), incubated at room temperature for 24 h, and then heated at 56 °C for 4 h. Atomic absorption measurements were performed by Dr. Frances W. J. Beck in the laboratory of Dr. Ananda Prasad at Wayne State University, School of Medicine (Detroit). Samples were diluted 1:10 and then 1:100 with zinc-free water immediately before analysis in a Varian SpectrAA-40 graphite furnace atomic absorption spectrophotometer equipped with a Zeeman background corrector. Duplicate or triplicate standards and samples (30 μL) were autoinjected into the furance, and zinc concentrations were calculated by a computerized program using known concentrations of zinc in bovine liver (National Bureau of Standards, Washington, D.C.) to establish the standard concentration curve. Interassay variation was no greater than 5%.

The DEPC-modified DHOase atomic absorption samples were prepared as follows. DEPC in an ethanol solution was added to recombinant CAD DHOase to a final concentration of 5 mM. An equivalent volume of ethanol was added to a control sample. After incubation for 1 h at room temperature, activity measurements of both samples indicated that the modified sample was 86% inactivated. *N*-Acetylhistidine was then added to both samples to a concentration of 20 mM. After 15 min of further incubation, EDTA was added to both samples to a concentration of 1 mM. Samples were chilled on ice, transferred to dialysis tubing, and dialyzed and processed as described earlier before injection into the spectrophotometer.

Zinc Blotting Experiments. $^{65}ZnCl_2$ in 0.5 M HCl was purchased from Dupont. Zinc blotting was performed according to the procedure of Schiff and co-workers (1988), with minor modifications. Nitrocellulose membranes containing electroblotted controls and samples were washed for 1 h in metal binding (MB) bufer (50 mM NaCl and 100 mM Tris-HCl, pH 8.0), incubated for 1 h with 200 μCi of $^{65}ZnCl_2$ in 75 mL of MB buffer, and washed four times with 100 mL of MB buffer. XAR film (Kodak) was exposed to plastic-wrapped membranes with an intensifying screen at −70 °C for several days.

RESULTS

Alignment of eukaryotic and prokaryotic sequences shows that five histidine and several acidic residues present in hamster CAD DHOase are conserved in other DHOases (Simmer et al., 1990). Histidine residues are implicated in catalysis (Christopherson & Jones, 1979, 1980) and, in addition, are proposed to participate in binding the single zinc ion present in the CAD DHOase monomer (Simmer et al., 1990; Kelly et al., 1986). In an attempt to identify the

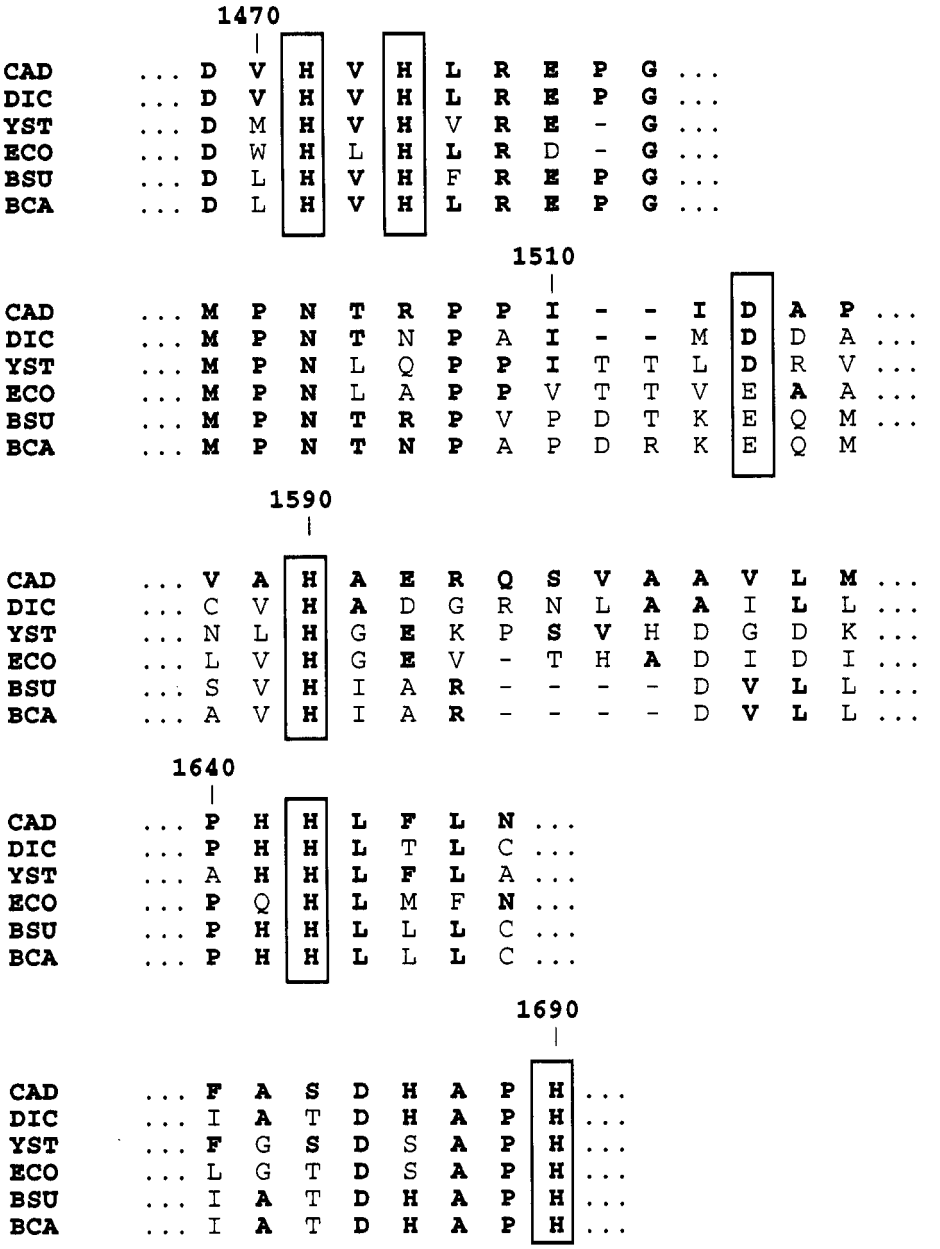

FIGURE 1: Alignment of regions containing conserved residues in the dihydroorotase region of hamster CAD. Regions of the dihydroorotase sequences of hamster (CAD (Simmer et al., 1990), *D. discoideum* (DIC) (Faure et al., 1989), *S. cerevisiae* (YST) (Guyonvarch et al., 1988), *E. coli* (ECO) (Backstrom et al., 1986), *B. subtilis* (BSU) (Quinn et al., 1991), and *B. caldolyticus* (BCA) (Ghim et al., 1993) were aligned according to Simmer and co-workers. Identical residues are shown in boldface print, and residues that were mutated by site-directed mutagenesis are boxed.

individual residues involved in these two functions, we have mutated one conserved acidic residue and each of the five conserved histidine residues in the recombinant CAD DHOase (Figure 1).

Complementation Experiments. Mutants H1471A, H1473A, H1590N, H1642N, H1690N, and D1512N were transformed into *E. coli* X7014, a DHOase-deficient strain. Cells transformed with the mutant D1512N consistently grew slightly better than cells transformed with pBZ22, the plasmid encoding the wild-type recombinant DHOase. Cells transformed with H1642N did not grow as well as cells transformed with pBZ22. Cells transformed with mutants H1471A, H1473A, H1590N, and H1690N did not grow, demonstrating that these mutants are unable to complement the host's deficiency (data not shown).

Protein Purification. CAD DHOase mutants were purified from EK1104 host cells in the same manner as the wild-type recombinant protein. The procedure consists of three steps: 45% ammonium sulfate precipitation of the sonicated cell extract, resuspension of the precipitate and dialysis, followed by salt gradient elution from a DEAE-Sephacel column (Zimmermann & Evans, 1993). The presence of mutant protein in column fractions was detected by Western blot with antibodies raised against the CAD DHOase domain.

Mutants in which a histidine residue was replaced by an asparagine residue, H1512N, H1590N, and H1690N, eluted at ~170 mM NaCl, higher in the gradient than the wild-type protein, which elutes at ~150 mM NaCl (Zimmermann & Evans, 1993). An SDS gel of the three mutants and the wild-type protein (Figure 2) shows that these preparations are suitable for use in DEPC modification experiments. Amino acid sequencing of H1690N gave the predicted N-terminal sequence, MLFVEALGQI. This preparation, like the wild-type recombinant preparations (Zimmermann &

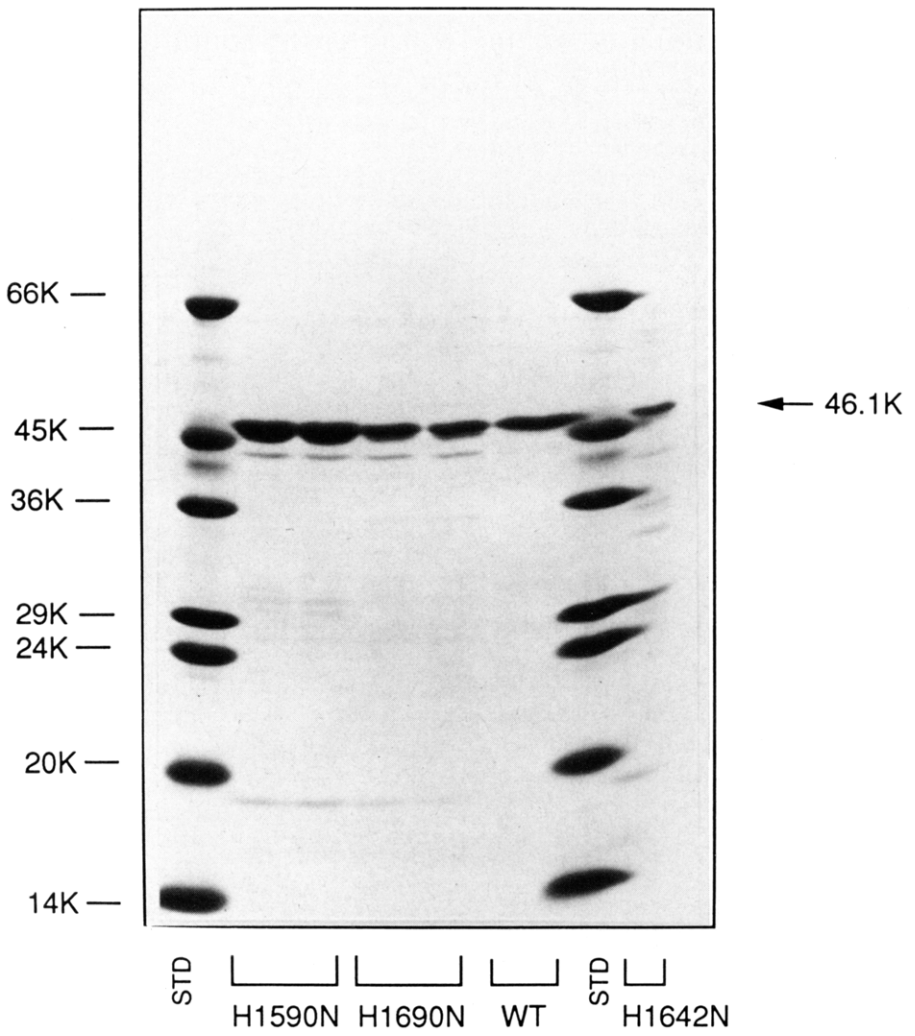

FIGURE 2: SDS−polyacrylamide gel electrophoresis of purified wild-type and mutant proteins. A linear gradient of 0−0.28 M NaCl was used to elute a DEAE-Sephacel column loaded with the dialyzed 45% ammonium sulfate precipitate of cell sonicate (see Experimental Procedures). Electrophoresis of 100 μL each of column fractions from a wild-type and three different mutant preparations are shown. The arrow indicates the calculated molecular weight of the recombinant proteins.

Evans, 1993), also contained a species that had three residues removed by an endogenous protease. Mutant D1512N was expressed at similar levels and eluted at the same salt concentration as the wild-type protein. Mutants in which a histidine residue was replaced by an alanine residue, H1471A and H1473A, eluted much later in the gradient, at ~270 mM NaCl. Western blots with the antibodies directed against *E. coli* DHOase[2] show that fractions eluting at this salt concentration are contaminated by small amounts of the endogenous enzyme.

Zinc Binding Studies. Atomic absorption experiments (Table 1) show that wild-type recombinant DHOase and mutant H1642N bind similar amounts of zinc. Mutants D1512N and H1590N bind one-fifth and one-third of the amount of zinc bound by the wild-type enzyme, respectively (Table 1). The lack of a full complement of zinc is likely to be the explanation for the inactivity of mutant H1590N. The preparation of samples for atomic absorption included extensive dialysis against solutions containing 10 mM EDTA to remove adventitiously bound zinc. This procedure does not affect the activity of wild-type enzyme and was used to demonstrate the presence of 1 g equiv of zinc per monomer of CAD DHOase (Kelly et al., 1986). Since mutant D1512N appears to be fully active, it presumably contains the catalytic zinc under normal activity assay conditions. The low zinc content of mutant D1512N measured by atomic absorption may be due to an increased sensitivity to chelators relative to the wild-type enzyme.

Table 1: Zinc Content of Wild-Type and Mutant DHOase Recombinant Proteins

protein	zinc bound (mole fraction)[a]
recombinant DHOase + DEPC	1.15 ± 0.18
D1512N	0.23 ± 0.01
H1590N	0.35 ± 0.01
H1642N	1.18 ± 0.23

[a] Data are expressed as mole fraction of the wild-type recombinant DHOase.

Zinc blotting is an alternative procedure that has been used to demonstrate the ability of proteins to bind zinc (Schiff et al., 1988). Proteins are first separated by SDS gel electrophoresis and then electrotransferred to nitrocellulose. The membrane is incubated with solutions containing radiolabeled zinc chloride, washed, and used to produce an autoradiogram. Enzymes known to contain zinc were employed as standards and exhibit a range of band intensities on autoradiograms

[2] Polyclonal antibodies directed against CAD DHOase do not cross react with *E. coli* DHOase.

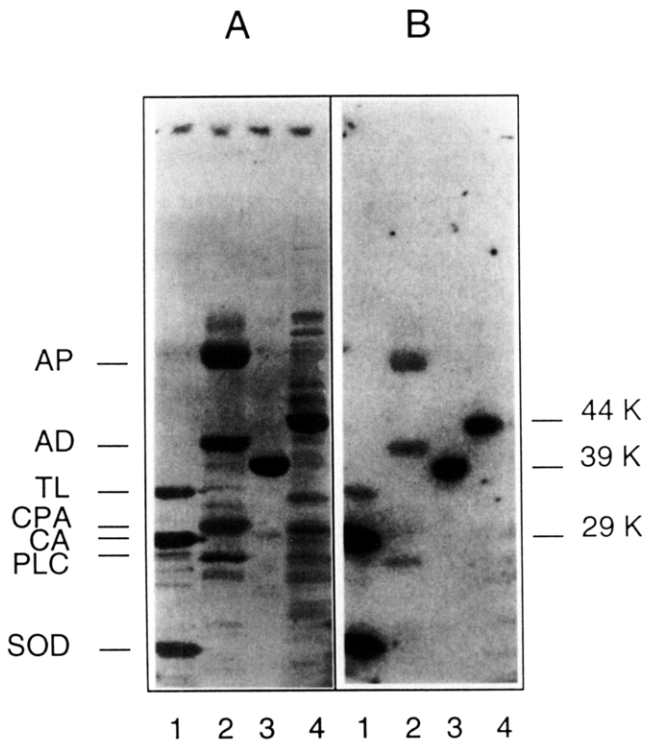

FIGURE 3: Zn^{65} binding to CAD and *E. coli* DHOases. Nitrocellulose membranes containing electrotransferred controls and samples were stained for protein (A) or incubated with $Zn^{65}Cl_2$. An autoradiogram prepared from the Zn^{65}-treated membrane is shown in panel B. Lane 1 was loaded with the zinc-containing proteins thermolysin (TL), carbonic anhydrase (CA), and superoxide dismutase (SOD); lane 2 was loaded with the zinc-containing proteins alkaline phosphatase (AP), alcohol dehydrogenase (AD), carboxypeptidase A (CPA) and phospholipase C (PLC); lane 3 contains *E. coli* DHOase; and lane 4 contains CAD DHOase proteolytic fragment. The molecular weights of CAD DHOase, *E. coli* DHOase, and carbonic anhydrase are indicated on the right side of panel B.

Table 2: Kinetic Parameters of CAD and the DHOase Domain-Catalyzed Formation of Carbamyl Aspartate from Dihydroorotate

protein	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})
CAD[a]	7.38 ± 1.02	1.52 ± 1.02	6.15 ± 0.36
proteolytic fragment[b]	21.8 ± 2.2	4.03 ± 0.16	2.96 ± 0.12
recombinant DHOase[c]	41.9 ± 3.5	3.63 ± 0.08	2.79 ± 0.06
D1512N	39.3 ± 5.6	7.24 ± 0.32	5.56 ± 0.25
H1590N	inactive		
H1642N	121.4 ± 10.0	0.419 ± 0.006	0.322 ± 0.005
H1690N	381.5 ± 58.6	0.107 ± 0.004	0.082 ± 0.003

[a] Data from Mally et al. (1981). [b] Data from Kelly et al. (1986). [c] Data from Zimmermann and Evans (1993).

(Figure 3). The most intense bands are those of carbonic anhydrase and superoxide dismutase, while weaker bands are observed for alcohol dehydrogenase, alkaline phosphatase, carboxypeptidase A, phospholipase C, and thermolysin. CAD DHOase and *E. coli* DHOase produce bands similar in intensity to carbonic anhydrase (Figure 3). Mutants H1471A and H1473A did not bind zinc under these conditions (data not shown).

Enzyme Kinetics. The kinetic parameters for purified mutant proteins, measured in the degradative direction at pH 8.3, are shown in Table 2. Mutant D1512N had a K_m similar to that of the wild-type recombinant. The k_{cat} of this mutant was approximately 2-fold higher than that of the wild-type recombinant and was similar to the turnover number measured for the dihydroorotase activity of the intact CAD molecule. Mutant H1642 exhibited 11.5% and mutant H1690N exhibited 2.9% of the activity of the wild-type recombinant DHOase. Both of these mutants had K_m values higher than that of the wild-type recombinant. H1642N had a K_m = 121.4 ± 10.0 μM (3-fold increase), and H1690N had a K_m = 381.5 ± 58.6 μM (9-fold increase). Mutant H1590N had no measurable activity. Kinetic parameters of mutants H1471A and H1473A were not determined because of persistent contamination by *E. coli* DHOase. Since these mutants fail to complement DHOase deficient cells and are unable to bind zinc on zinc blots, they are presumably inactive.

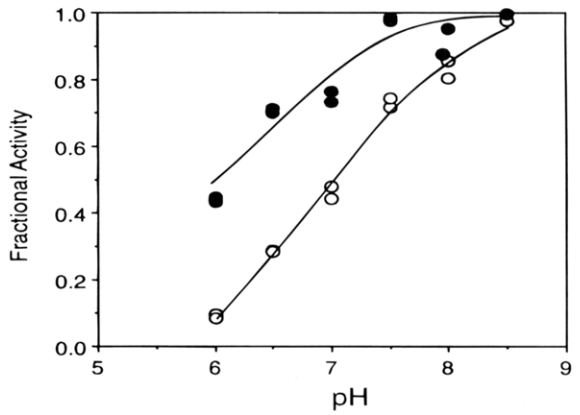

FIGURE 4: Effect of pH on the degradative activity of wild-type and mutant CAD DHOases. Activity of mutants H1642N (○) and H1690N (●) was determined by the colorimetric assay (Prescott & Jones, 1983) using 0.5 mL incubation mixtures buffered with 100 mM HEPES (pH 7.0, 7.5, 8.0) or 100 mM MES (pH 6.5, 7.0). Reactions were initiated by adding 100 μL (~3 μg) of mutant protein, incubated at 37 °C for 60 min, and the quenched.

pH Studies. The work of Christopherson and Jones (1979) showed that the DHOase activity of mouse CAD is pH dependent in both the degradative and the biosynthetic directions. In the degradative direction, they found the enzyme to be 17-fold more active at pH 8.0 than at pH 6.0. We observe a similar dependence of activity on pH with the recombinant hamster CAD DHOase, although the magnitude of the difference is smaller. To determine whether either histidine 1642 or 1690 could play a role in the pH dependence, we measured the degradative activity of the corresponding mutants between pH 6.0 and 8.0. The activity of mutant H1642N exhibits a pH dependence similar to that of the wild-type recombinant, while the pH dependence of the activity of H1690N is greatly reduced. Only a 2.3-fold difference was found in the activities of H1690N measured at pH 6.0 and 8.0, compared to an 11-fold difference in the activities of H1642N measured at these same pH values (Figure 4).

Diethyl Pyrocarbonate Inactivation. Christopherson and Jones (1979) reported that the DHOase activity of mouse CAD was inhibited by DEPC and that the substrate protected the enzyme against inactivation. We observe similar protection against inactivation by dihydroorotate with both the hamster CAD DHOase proteolytic fragment and the recombinant protein. We have used two types of experiments, one spectrophotometric and the other based on radiolabeling, to determine the number of residues modified by DEPC.

The N-carbethoxylation of histidine residues by DEPC is accompanied by an increase in absorbance between 230 and

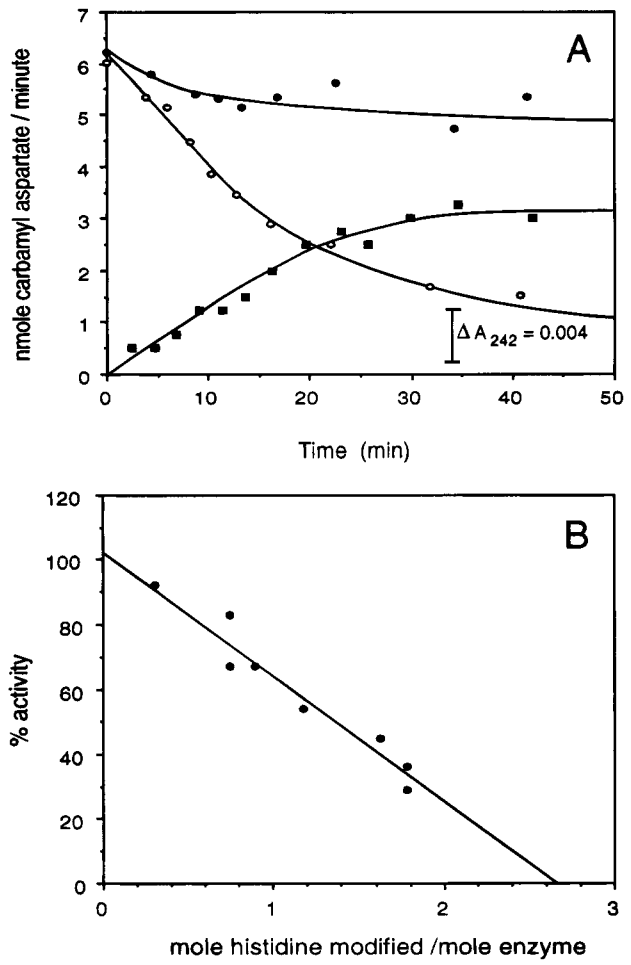

FIGURE 5: Inactivation of CAD DHOase proteolytic fragment with diethyl pyrocarbonate. Panel A: Matched cuvettes containing equal volumes (0.59 mL each) of 2.0 μM purified CAD DHOase proteolytic fragment in buffer (50 mM HEPES, 150 mM NaCl, and 5% glycerol, pH 7.1) were placed at 24 °C in thermostated cuvette holders in the reference and sample beams of the spectrophotometer. DEPC in ethanol was added to the sample cuvette to a final concentration of 1 mM, and an equivalent volume of ethanol was added to the reference cuvette. During the next 45 min, absorbance was scanned from 300 to 200 nm, and 2 μL aliquots were withdrawn for activity measurements. Symbols: (○) activity of sample; (●) activity of reference; (■) ΔA_{242nm}, absorption difference between sample and reference. Panel B: Percent of activity plotted as a function of the number of moles of histidine residues modified per mole of CAD DHOase. At 100% inactivation, 2.6 mol of histidine per mole of enzyme is modified.

250 nm (Miles, 1977). The number of modified residues can be calculated by using $\Delta\epsilon_{242nm} = 3900\ M^{-1}\ cm^{-1}$ (Choong et al., 1977). Upon the addition of DEPC to the DHOase proteolytic fragment, we observed an initial rapid increase in absorbance (within the time required to mix the solutions), corresponding to the modification of approximately 12 of 18 total histidine residues. There was no loss of DHOase activity during this phase of the reaction. The inactivation of the enzyme correlated with a more gradual increase in absorbance (Figure 5A), which followed the initial rapid reaction with DEPC. Figure 5B shows that 2.6 histidine residues are carbethoxylated during complete inactivation of CAD DHOase proteolytic fragment. No decrease in absorbance at 278 nm was observed during the experiment, indicating that O-carbethoxylation of tyrosyl residues does not occur (Miles, 1977). Addition of the metallochromic indicator PAR to DEPC-inactivated CAD

Table 3: Radiolabeling of Wild-Type and Mutant DHOase Recombinant Proteins with [*carbonyl*-^{14}C]Diethyl Pyrocarbonate[a]

protein	nmol incorporation/nmol DHOase	
	−substrate	+substrate
recombinant DHOase	9.5 ± 2.1	7.6 ± 1.6
H1590N	10.5 ± 3.3	8.6 ± 2.3
H1642N	10.1 ± 1.8	8.3 ± 1.5
H1690N	7.9 ± 1.6	8.0 ± 1.4

[a] Radiolabeling methods are described in the Experimental Procedures.

DHOase shows that zinc is not released during histidine modification (data not shown). This is consistent with atomic absorption experiments, which show that DEPC-modified recombinant DHOase contains the same amount of zinc as the unmodified enzyme (Table 2). Hydroxylamine is commonly used to reactivate DEPC-treated enzyme (Miles, 1977). This reagent appears to inhibit CAD DHOase and, thus, could not be used to remove the carbethoxy group from the modified histidine residues (B. H. Zimmermann and D. R. Evans, unpublished observations).

Table 3 shows the results of radiolabeling experiments. In the absence of substrate, 9.5 ± 2.1 residues of the wild-type recombinant DHOase are labeled. When 5 mM dihydroorotate is present in the DEPC incubation mixture, only 7.6 ± 1.6 residues are labeled, indicating that approximately 2 residues are protected by substrate. The incorporation of radiolabel is similar to that observed for the wild-type enzyme for two of the mutants. In the case of mutant H1590N, 10.5 ± 3.3 residues are labeled without the substrate, while 8.6 ± 2.3 residues are labeled in the presence of substrate. For mutant H1642N, 10.1 ± 1.8 residues are labeled without the substrate, while 8.3 ± 1.5 residues are labeled in the presence of substrate. Mutant H1690N behaved differently. Essentially the same amount of radiolabel is incorporated whether or not substrate is present: 7.9 ± 1.6 residues are labeled in the absence of substrate and 8.0 ± 1.4 residues are labeled in the presence of substrate.

DISCUSSION

Catalytic zinc ions in the active sites of enzymes are coordinated by a combination of three histidine, glutamate, aspartate, or cysteine residues (Brown et al., 1983). Vallee and Auld (1990) have surveyed the zinc ligands in 12 enzymes with known crystal structures. They report that this diverse group of enzymes, which includes dehydrogenases, lyases, and hydrolases, exhibits a characteristic spacing of residues that bind the zinc. The first and second zinc-coordinating residues are separated by 1−3 residues in the primary sequence, while the second and third zinc-coordinating residues are separated by a longer spacer ranging in length from 20 to 120 residues. Our sequence alignment of monofunctional dihydroorotases (*Escherichia coli*, *Salmonella typhimurium*, and the *Saccharomyces cerevisiae ura4* gene) and the dihydroorotase domains of multifunctional enzymes (CAD, *Drosophila melanogastor*, and *Dictyostelium discoideum*) identified several conserved histidine and acidic residues, but showed no conserved cysteine residues in the CAD DHOase domain (Simmer et al., 1990). The spacing of histidines 1471 and 1473 and glutamate 1512 initially suggested that these conserved residues might be the ligands coordinating the zinc ion in CAD.

Site-directed mutation studies of various enzymes containing catalytic zinc ions indicate that replacement of the zinc-chelating residues usually results in mutant proteins with little or no activity. For example (Le Moual et al., 1993), when either of the two proposed histidine zinc ligands of neutral endopeptidase 24.11 are replaced with other amino acids (cysteine, aspartate, glutamate, or tyrosine), the resulting mutant enzymes are inactive. Replacement of glutamate, the third ligand, by either aspartate or cysteine results in mutant proteins with 5−6% of the wild-type activity and ~5-fold greater sensitivity to chelator. Replacement of the same glutamate with histidine, tyrosine, or serine leads to inactive mutant enzymes. Another example of this type of study was reported by Medina and co-workers (1991), who constructed mutations of each of the three residues that are proposed to bind zinc in leukotriene A_4 hydrolase. The mutant enzymes H295Y, H299Y, and E318Q are inactive, and atomic absorption measurements show that they contain no significant amounts of zinc. A final example is the site-directed mutagenesis study of adenosine deaminase (Bhaumik et al., 1993), an enzyme in which three histidines were identified by X-ray crystallography as zinc ligands (Wilson et al., 1991). Mutant proteins of one zinc ligand, H214A, H214N, and H214L, exhibit variable activities ranging from 0.01% to 0.1% of the wild type, depending on whether zinc is present during purification. H17A, a mutant protein of a second zinc ligand, exhibits 0.1−3% of the wild-type activity and is unstable. Although atomic absorption measurements indicate that mutants H214A and H214N retain the zinc ion, it should be noted that these samples were prepared in the absence of zinc chelators.

The zinc ligands of the dihydroorotase are not yet unequivocally identified. However, we can conclude the following. Since mutant D1512N has kinetic parameters similar to those of the wild-type recombinant, glutamate 1512 is unlikely to participate in binding zinc. Mutants H1471A and H1473A fail to complement a dihydroorotase-deficient *E. coli* strain and, thus, appear to be inactive. This inactivity is likely to be related to the inability of these mutants to bind zinc. Furthermore, no measurable activity and low quantities of zinc were found for purified mutant H1590N. On the basis of spacing in the primary sequence and the activity and zinc binding of recombinant proteins in which these residues have been mutated, the candidates for zinc ligands are His1471, His1473, and His1590.

An important feature of enzymes containing catalytic zinc was identified by Christianson and Alexander (1989) after an analysis of crystal structures. The zinc binding domains examined all contain a carboxylate−histidine−zinc triad interaction that is proposed to enhance metal complexation or to modulate the pK_a of any metal-bound water molecule (Christianson & Alexander, 1989). Further experimentation will be required to establish whether DHOases contain such a triad and, if so, to identify the carboxylate. We favor the idea, suggested by Brown and Collins (1991), that one of the conserved acidic residues in the region that includes histidines 1471 and 1473 may be involved.

Christopherson and Jones (1979) showed that DEPC inactivates mouse DHOase and that substrate protects the enzyme from inactivation. In this work, we determined the number of residues in the enzyme modified by DEPC and attempted to identify specific residues involved. Both spectroscopic studies and [^{14}C]DEPC incorporation demonstrated that the loss of activity is associated with the modification of approximately two histidine residues located at or near the active site. We have used two approaches to demonstrate that CAD DHOase modified by DEPC still binds zinc. The first was a method used by Shang and co-workers (1989) to study *Xenopus* transcription factor IIIA (TFIIIA), a protein in which zinc is proposed to be coordinated by cysteine and histidine residues. When TFIIIA is treated with either a histidine modification reagent, DEPC, or a cysteine modification reagent, *p*-(hydroxymercuri)benzenesulfonate, zinc dissociates from the protein and can be detected in solution by the metallochromic indicator PAR. Our experiments with PAR indicate that DEPC modification of DHOase does not release the bound zinc. In the second approach, the zinc content of DEPC-modified recombinant DHOase was measured by atomic absorption. We observed that both modified and unmodified enzymes contain the same amount of zinc. These results imply that the residues modified by DEPC during inactivation are not the same residues that are involved in coordination of the catalytic zinc ion. According to our hypothesis, residues 1471, 1473, and 1590 therefore are not modified by DEPC. The results of the substrate protection experiments with radiolabeled DEPC obtained for mutant H1590N are in accordance with this idea. Like mutant H1590N, mutant H1642N exhibits essentially the same radiolabeling behavior as the wild-type protein. In this case we conclude that residue 1642 is not close enough to the active site to be protected by the presence of substrate. This is consistent with the observation that replacement of this residue results in a mutant protein that retains a substantial amount of activity (~12% of wild type).

Replacement of histidine 1690 results in a mutant enzyme (H1690N) of low activity (~3% of wild type) that is no longer protected against DEPC modification by the substrate. This residue is thus likely to be one of the two whose modification results in enzyme inactivation. It is interesting to note that replacement of this single residue eliminates DEPC incorporation into two sites. It may be that its carbethoxylation results in structural changes that expose a second, otherwise unreactive histidine residue. If this is the case, only one histidine, residue 1690, may be essential for catalysis. Another possibility is that mutagenesis of residue 1690 yields a mutant protein in which the second histidine is inaccessible to DEPC.

The importance of histidine 1690 in catalysis is reinforced by the pH study (Figure 4), which showed that this residue is responsible, at least in part, for the pH dependence of the degradative reaction. Christopherson and Jones (1980) have proposed a model in which the DHOase exists in four protonation states. They find that the pH behavior of the degradative reaction is best explained if dihydroorotate binds equally well to the two most basic forms of the enzyme. In this scenario, histidine 1690 would be one of the functional groups that is deprotonated in these two catalytically active species.

ADDED IN PROOF

We have constructed and expressed the mutant protein H1471N. This mutant exhibits purification properties similar to those of the wild-type recombinant, and it can be isolated without contamination by *E. coli* DHOase, in contrast to the mutant protein H1471A. We observe that purified H1471N

has no measurable activity, a result that is consistent with the failure of H1471A to complement DHOase-deficient host cells.

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