Mutagenesis of Histidinol Dehydrogenase Reveals Roles for Conserved Histidine Residues†

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Received November 19, 1998; Revised Manuscript Received March 16, 1999

ABSTRACT: The dimeric zinc metalloenzyme L-histidinol dehydrogenase (HDH) catalyzes an unusual four-electron oxidation of the amino alcohol histidinol via the histidinaldehyde intermediate to the acid product histidine with the reduction of two molecules of NAD. An essential base, with pK_a about 8, is involved in catalysis. Here we report site-directed mutagenesis studies to replace each of the five histidine residues (His-98, His-261, His-326, His-366, and His-418) in *Salmonella typhimurium* with either asparagine or glutamine. In all cases, the overexpressed enzymes were readily purified and behaved as dimers. Substitution of His-261 and His-326 by asparagine caused about 7000- and 500-fold decreases in k_{cat}, respectively, with little change in K_M values. Similar loss of activity was also reported for a H261N mutant *Brassica* HDH [Nagai, A., and Ohta, D. (1994) *J. Biochem. 115*, 22−25]. Kinetic isotope effects, pH profiles, substrate rescue, and stopped-flow experiments suggested that His-261 and His-326 are involved in proton transfers during catalysis. Sensitivity to metal ion chelator and decreased affinities for metal ions with substitutions at His-261 and His-418 suggested that these two residues are candidates for zinc ion ligands.

In catalysis by coenzyme-linked dehydrogenases, hydride transfer to coenzyme is coupled to proton transfer from substrate to an active-site base. Although the order and degree of coupling between the two transfers is still not fully understood, it may vary from concerted transfer, which was proposed as a general rule (*1*), to sequential proton and hydride transfer proposed for ADH[1] (*2*, *3*). Investigations of catalytic mechanisms in dehydrogenases have enabled the identification of active-site base residues which include histidine (*4*−*16*), lysine (*17*, *18*), cysteine (*19*), glutamate (*20*), aspartate (*21*), and tyrosine (*22*−*24*).

The four-electron oxidoreductases catalyze both alcohol and aldehyde oxidations at a single active site with sequential reduction of two molecules of coenzyme. Investigation of proton transfer in this class of enzymes must address an additional problem, which is whether the same base is used twice in the overall reaction or whether two distinct residues serve as active-site bases during the alcohol and aldehyde oxidation half-reactions. In the four-electron oxidoreductase HMGR, data from X-ray structure and mutagenesis have shown that an aspartate protonates both HMG and the mevaldate intermediate, and a histidine residue catalyzes proton transfer at the coenzyme A thiol (*25*−*28*).

L-Histidinol dehydrogenase (HDH; EC 1.1.1.23) catalyzes the four-electron oxidation of the amino alcohol histidinol to the acid product histidine with two sequential reductions of NAD. The proposed intermediate histidinal has been well documented (*29*−*33*). By analogy with HMGR, one can envision a mechanism for HDH, in which the same enzymic base residue is used during the first (histidinol oxidation) and second (histidinal oxidation) half-reactions; whereas another base residue is involved in the activation of attacking nucleophile. In the preceding paper, we have analyzed k_{cat} and k_{cat}/K_M versus pH profiles of *Salmonella typhimurium* HDH and identified a catalytically essential enzymic base with pK_a between 7.9 and 8.4 (*34*). On the basis of their pK_a values and degree of conservation, we hypothesized that histidine residues in HDH might serve as enzymic bases during the two half-reactions. Although the reported pK_a for the enzymic base in HDH is higher than the solution pK_a of histidine imidazole (*35*), pK_a values for enzymic groups can be perturbed by 2 pH units or more; depending upon the microenvironment within the active site and the stickiness of the substrate (*36*−*38*).

Grubmeyer et al. (*39*) reported that *S. typhimurium* HDH is a zinc metalloenzyme. Nagai and Ohta (*40*) have interpreted their mutagenesis studies to indicate that His-261 in cabbage *Brassica oleracea* HDH is a ligand for a catalytic zinc. Substitutions at two other conserved histidine residues of the cabbage enzyme, His-94 and His-367, did not cause loss of enzymatic activity. Later work by Kanaori et al. (*41*) suggested that the zinc ion in *Brassica* HDH is liganded to a combination of nitrogen (histidine) and oxygen (aspartate) ligands. This potential role for histidine residue is not

† This work was supported by the National Institute of Health.

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[1] Abbreviations: ADH, alcohol dehydrogenase; EPR, electron paramagnetic resonance; HDH, L-histidinol dehydrogenase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); histidinal, L-histidinaldehyde; HMG, 3-hydroxy-3-methylglutaryl coenzyme A; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; KIE, kinetic isotope effects; PAR, 4-(2-pyridylazo)resorcinol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; WT, wild-type histidinol dehydrogenase.

10.1021/bi982758p CCC: $18.00
Published on Web 05/14/1999

Table 1: Oligonucleotides Used in Construction of Histidine Mutants

mutation	primer[a]
H98N	5′ ACGTTCAATTCCGCGCAGA 3′
H261N	5′ AGGCTGAGAACGGCCCGGA 3′
H326N	5′ GCCGGAAAACTTAATCA 3′
H366N	5′ AACCAACAATGTTTTACC 3′
H418Q	5′ GACCGCCCAAAAAAATGCCG 3′

[a] Mutated codons are underlined.

unexpected, since histidine is the most common ligand for Zn^{2+} among zinc metalloenzymes (*42*, *43*). The mutagenesis studies by Nagai and Ohta (*40*) on cabbage HDH, as well as the common use of histidine as active-site base, led us to investigate possible roles of histidine residues in *S. typhimurium* HDH in catalysis and metal ligation.

In this report, we investigate the roles of all five conserved histidine residues in *S. typhimurium* HDH using site-directed mutagenesis. Our results revealed that His-261 and His-326 are involved in proton transfers and His-261 and His-418 are candidates for zinc ion ligands.

MATERIALS AND METHODS

Biochemicals. Biochemicals and bacterial strains used were described in Teng et al. (*44*) and in the preceding paper in this issue (*34*). Hepes was from Research Organics. Chelex-100 (sodium form, 200−400 mesh) was from Bio-Rad. 1,10-Phenanthroline was from J. T. Baker Chemical Co. Manganese chloride (tetrahydrate) and zinc chloride were Aldrich Gold Label grade. PAR was from Eastman Kodak and was a gift of Dr. David E. Ash, Temple University School of Medicine.

Mutagenesis. Site-directed mutagenesis was performed according to Kunkel (*45*) with the Bio-Rad Muta-Gene Version 2 Mutagenesis Kit as described in Teng et al. (*44*). Oligonucleotides used in mutagenesis are listed in Table 1. All mutants were fully sequenced by single-stranded DNA sequencing using Sequenase Version 2.0 (United States Biochemical) to ensure that no secondary mutation had occurred.

Overexpression and Purification of Mutant Enzymes. Mutated *hisD* genes were expressed using the T7 RNA polymerase/promoter system in the *Escerichia coli* strain *BL21*(*DE3*) as described in Teng et al. (*44*). Two of the mutant enzymes, carrying the H261N and the H326N mutations, show low activity and were expressed in the *E. coli hisD*$^-$ strain *his755* (ref *46*; a gift of Dr. Philip Hartman, The Johns Hopkins University). Strain *his755* was first transformed with plasmid pGP1−2 (a gift of Dr. Stanley Tabor, Harvard Medical School), which encodes the T7 RNA polymerase and confers kanamycin resistance (*47*). Colonies harboring pGP1−2 were then transformed with plasmid pHT261N or pHT326N (carrying the H261N or the H326N mutation, respectively) and screened on LB plates supplied with 50 μg/mL of ampicillin and 75 μg/mL of kanamycin. Overexpression of mutant enzyme was achieved by increasing the incubation temperature from 30 to 42 °C, which induced the production of T7 RNA polymerase. Cells were harvested after 3 h of induction and stored in buffer E [20 mM Tris, 20 mM succinic acid (pH 6.2), 0.1 mM $MnCl_2$] supplemented with 8% glycerol as described in Teng et al. (*44*). Expression of mutant enzymes was monitored with SDS−PAGE (*48*).

Procedures for purification and crystallization were carried out as described in Teng et al. (*44*) in buffer E supplemented with 8% glycerol. For long-term storage of mutant enzymes, crystalline enzyme was dialyzed against buffer E supplemented with 50% glycerol and stored refrigerated in the same buffer.

Analytical Gel Filtration Chromatography. Gel filtration chromatography was performed with a Pharmacia-LKB Superdex 200 HiLoad column (1.6 × 60 cm) preequilibrated with buffer E supplemented with 8% glycerol. Enzyme (150 μg) was chromatographed at a flow rate of 1 mL/min at room temperature.

Thermal Stability of WT versus Mutant Enzymes. WT and mutant enzymes were dissolved in buffer containing 50 mM sodium glycine (pH 9.0), 0.5 mM $MnCl_2$, and 8% glycerol, followed by incubation at 60 °C. Samples were withdrawn at various times and assayed in standard assay buffer.

Enzyme Assays. Enzymes were assayed for HDH activity according to Loper (*49*). The standard assay buffer contained 50 mM sodium glycine (pH 9.0), 0.5 mM $MnCl_2$, 2 mM histidinol, and 10 mM NAD. Crystalline enzyme was centrifuged and the pellet dissolved in buffer containing 50 mM sodium glycine (pH 9.0), 0.5 mM $MnCl_2$, and 8% glycerol. As necessary, enzymes were desalted by passing through centrifuge columns (*50*) equilibrated with 50 mM sodium glycine (pH 9.0), 0.5 mM $MnCl_2$, and 8% glycerol. Routine assays were performed by adding enzyme solution to assay buffer (1 mL) containing 50 mM sodium glycine (pH 9.0), 0.5 mM $MnCl_2$, and 10 mM NAD, and equilibrated at 30 °C for 5 min. Histidinol (final concentration 2 mM) was added to start the reaction. With mutant enzymes exhibiting low activities, assays were performed against blanks containing the above components but lacking histidinol.

Stoichiometry experiments, K_M measurements, and nondenaturing polyacrylamide gel electrophoresis were conducted according to Teng et al. (*44*).

Equilibrium Binding of Histidinol with WT and H261N. Overnight equilibrium binding of [^{14}C]histidinol with WT was conducted with the Microvolume Dialyzer (Pharmacia). Substrate binding experiments with H261N were conducted using equilibrium gel filtration as described in Grubmeyer et al. (*51*). The enzyme concentrations used for WT and H261N were 79 and 98 μM subunit, respectively. With H261N, peaks of radioactivities were used to calculate the amount of bound substrate. Data were analyzed by computer (*52*) to obtain the K_D values for histidinol and numbers of binding sites.

Kinetics with Alternative Substrate. Imidazolyl propanediol was synthesized according to Grubmeyer et al. (*53*). For k_{cat} determinations, the substrate was present at 11 μM to 2 mM, and NAD was at 10 or 20 mM. For K_M determinations with imidazolyl propanediol, the substrate was present from 11 μM to 2 mM, and NAD was present at 10 mM. K_M values for NAD were determined with 0.2−10 mM coenzyme, and with 2, 2, 10, and 5.8 mM imidazolyl propanediol for WT, H326N, H366N, and H418Q, respectively.

Deuterium Kinetic Isotope Effects. Diprotiated (H,H), monodeuterated (H,D; 58% substitution, 42% dideuterated) and dideuterated (D,D)histidinol were synthesized according to Grubmeyer et al. (*53*). Kinetic deuterium isotope effects (^DV) were measured with 0.2 mM (H,H), (H,D), and (D,D)-

histidinols. $^{D}V/K$ KIE with histidinol were determined with 2−50 μM (H,H) and (D,D) histidinols, with NAD present at 10 mM.

Substrate Rescue Experiments. Mutant HDH H261N (669 μg, 14.5 nmol of subunit) or H326N (481 μg, 10.5 nmol of subunit) were incubated in assay buffer (1 mL) containing 50 mM sodium glycine (pH 9.0), 0.5 mM $MnCl_2$, and 10 mM NAD, in a quartz cuvette, and allowed to temperature equilibrate for 5 min in the 30 °C thermostated holder of a Perkin-Elmer 552A spectrophotometer. Histidinol (5 μL of a 1 mM stock) was added to start the reaction. NADH production was monitored by absorbance at 340 nm. At indicated times, 1.5 μg (0.03 nmol of subunit) or 7.5 μg (0.16 nmol of subunit) of WT was added to the reaction mixture containing H261N or H326N, respectively. NADH production profiles with WT, H261N, and H326N were obtained individually for comparisons.

Pre-Steady-State Kinetics. Pre-steady-state kinetic measurements with H326N were conducted with a Hi-Tech Scientific SF-61 stopped-flow spectrophotometer as described in the preceding paper in this issue (*34*). All buffers used contained 50 mM sodium glycine (pH 9.0) and 0.5 mM $MnCl_2$. In the experiments described, the enzyme syringe contained 192 μM enzyme subunit preincubated with 4 mM (H,H)histidinol, and the coenzyme syringe contained 20 mM NAD. Results reported are the means of five injections.

pH Profiles. For log(k_{cat}) pH profiles with H98N, H366N, and H418Q, enzyme crystals were dissolved in buffer containing 50 mM sodium glycine, 50 mM sodium Hepes (pH 9.0), 0.5 mM $MnCl_2$, and 8% glycerol. Enzyme solution was added to assay buffer (1 mL) containing 50 mM glycine, 50 mM Hepes, 0.5 mM $MnCl_2$, and 10 mM NAD, titrated to desired pH values with NaOH and allowed to incubate at 30 °C for 5 min. An aliquot (10 μL) of 0.2 M histidinol was added to start the reaction. For log(k_{cat}/K_M) pH profiles of histidinol with H261N and H326N, enzyme stock stored in buffer E supplemented with 50% glycerol were used. Assays were performed as described above except histidinol concentration used ranged from 1 μM to 1 mM. Data were analyzed by the program HA-BELL (*52*) to determine pK_a values.

Chelator Sensitivity and Inactivation/Reactivation Measurements. Chelator sensitivity experiments were conducted according to Teng et al. (*44*). WT and mutant enzymes were dissolved in buffer containing 50 mM sodium glycine (pH 9.0), 0.1 mM $MnCl_2$, and 8% glycerol. After incubation at 30 °C for 5 min, the enzyme was assayed for activity either in standard assay buffer or in buffer in which 0.5 mM $MnCl_2$ was replaced by 1 mM sodium EDTA.

Inactivation/reactivation experiments were conducted according to Teng et al. (*44*). Crystalline enzyme was dissolved in 50 mM Tris-Cl (pH 8.0) supplemented with 8% glycerol and a sample was assayed in standard assay buffer. The dissolved enzyme was allowed to incubate at 30 °C for 5 min in the presence of 1 mM 1,10-phenanthroline and 10 mM sodium EDTA before determining activity in standard assay buffer with 1 mM sodium EDTA replacing 0.5 mM $MnCl_2$. Enzyme exposed to this procedure was designated "inactivated enzyme". To remove 1,10-phenanthroline and EDTA, samples were passed through centrifuge columns (*50*) preequilibrated with buffer containing 50 mM Tris-Cl (pH 8.0) and 8% glycerol. To produce "reactivated" enzyme, $MnCl_2$ was added to the eluate at 1 mM final concentration, followed by incubation at 30 °C for 5 min, before assayed in standard assay buffer.

Metal Ion Content. To determine enzyme-bound zinc ion concentration, the chromophoric compound PAR was employed (*54*). In generating "storage" conditions, WT and mutant enzymes stored as ammonium sulfate crystals (or in glycerol) were dissolved (or diluted) in 20 mM Tris-succinate (pH 6.0) supplemented with 0.1 mM $MnCl_2$. Enzyme solutions were passed through centrifuge columns (*50*) preequilibrated with Tris-succinate (pH 6.0) to remove ammonium sulfate or glycerol. To the eluates was added $MnCl_2$ to 0.1 mM, followed by incubation at 30 °C for 5 min. The samples were then loaded on second centrifuge columns preequilibrated with 0.5 M sodium Hepes (pH 7.0). From each sample, an aliquot of the eluate from the second centrifuge column was withdrawn to determine protein concentration. To the remaining eluate was added an equal volume of 6% perchloric acid, followed by boiling for 1 min to ensure complete denaturation of the enzyme. The sample was then centrifuged to remove denatured protein. A 50 μL sample of the supernatant was then added to 950 μL of 0.1 mM PAR solution prepared in 0.5 M sodium Hepes (pH 7.0). Zinc ion concentrations were determined by absorbance at 500 nm by comparing to a standard curve. The same samples were also used to determine free manganese ion contents by EPR spectroscopy at 27 °C with a Bruker ER 200D-SRC electron paramagnetic resonance spectrometer operating at 9.7 GHz with the center field set at 3400 G. The samples were contained in quartz tubings (inside diameter 0.4 mm, outside diameter 2 mm), and each sample was scanned once with a relative gain of 2.52×10^4. Manganese ion concentrations in the samples were determined by comparing to a standard curve. For determining zinc and manganese ion concentrations under "assay" conditions, the enzymes were prepared similarly, except Tris-succinate was replaced by 50 mM sodium glycine (pH 9.0), and 0.1 mM $MnCl_2$ was replaced by 0.5 mM $MnCl_2$. All buffers used in metal content measurements were rendered metal ion free by passing through Chelex-100 column. Control samples prepared without enzyme did not show detectable levels of zinc and manganese ions.

RESULTS

Mutagenesis of Histidine Residues. Primary sequence comparisons of HDHs between Gram-positive, Gram-negative, and archaea bacteria, yeast, fungus, and cabbage (*55*−*62*) using the program BLAST (*63*) revealed that all five histidine residues (His-98, His-261, His-326, His-366, and His-418) in *S. typhimurium* HDH are conserved in all cases. To investigate the possible roles of these conserved histidine residues, site-directed mutagenesis was employed to mutate His-98, His-261, His-326, and His-366 to asparagine and His-418 to glutamine.

Purification of mutant enzymes to apparent homogeneity was carried out as described in the Materials and Methods. All mutant enzymes were isolated in high yield (20−30 mg/L of culture) and formed microcrystals.

Physical Characterization of Mutant Enzymes. Nondenaturing gel electrophoresis of all mutant enzymes showed single bands with the same mobility as WT, indicating that

Table 2: Steady-State Kinetics of Histidine Mutants

enzyme	K_M histidinol (μM)	K_M NAD (mM)	k_{cat} (s^{-1})	stoichiometry, NADH/histidinol	^DV[b]	$^DV/K$[b]	^DV (H,D) histidinol
WT	15.0 ± 2.4	1.1 ± 0.2	13.0	2.0	2.6 ± 0.1	1.3 ± 0.1	2.2
H98N	15.6 ± 8.3	0.6 ± 0.1	3.3	2.0	2.0	nd	nd
H261N	31.1 ± 5.5	2.1 ± 0.3	0.0019	1.9[a]	2.0 ± 0.3	1.5 ± 0.2	1.5
H326N	1.7 ± 0.5	0.9 ± 0.1	0.025	1.9	0.9 ± 0.1	0.9 ± 0.2	nd
H366N	111.9 ± 10.5	1.3 ± 0.1	4.9	2.1	2.0	nd	nd
H418Q	16.5 ± 7.6	15.3 ± 4.0	0.58	2.1	2.6	nd	nd

[a] Stoichiometry reported was determined with a molar excess of enzyme over substrate. [b] Measured with (D,D)histidinol. nd, not determined.

the mutant enzymes were structurally intact. All proteins behaved as dimers on Superdex 200, with elution volumes of peak and peak widths identical to WT (not shown). Thermal stability experiments showed that, after 60 min of incubation at 60 °C, WT retained 61% of its original enzymatic activity. Similar results were also observed for H326N and H366N (65 and 64% remaining activity, respectively). H98N and H261N showed greater instability, with 32 and 37% remaining activity, respectively. H418Q showed substantial thermal instability compared to WT, being almost completely inactivated (3% remaining activity) after 15 min of incubation.

Steady-State Kinetics of Mutant Enzymes. Table 2 shows the steady-state kinetic parameters of WT and mutant enzymes. H98N and H366N were least affected, giving k_{cat} values 4- and 3-fold lower than WT (13.0 s^{-1}), respectively. Nagai and Ohta (*40*) reported no loss of activity when the analogous His-94 and His-367 in *Brassica* HDH were mutated to asparagines. With H98N, the K_M values for either histidinol or NAD were not perturbed, whereas H366N exhibited a 7.5-fold increase in K_M for histidinol. For H418Q, k_{cat} was 22-fold lower than WT, and K_M for NAD had increased by 14-fold when compared to WT. H326N showed a k_{cat} value 512-fold lower than WT, and a 9-fold decrease in K_M for histidinol. The least active mutant enzyme, H261N, catalyzed the HDH reaction with $k_{cat} = 0.0019$ s^{-1} (6800-fold lower than WT), but the K_M values for both histidinol and NAD were similar to WT. No increase in activity was observed when up to 200 mM sodium glycine or 50 mM glycylglycine was added to assays with WT, H261N, and H326N (not shown).

The extremely low activity for the H261N and H326N enzymes raised concern as to whether those activities were truly enzymatic. However, in each case, the reaction was strictly dependent on the presence of both substrates, was directly proportional to the amount of enzyme added, and was inhibited by imidazole (results not shown), an inhibitor of HDH (*53*). In addition, expression of these mutant enzymes in a *hisD*$^-$ deleted host obviated concern that contamination by host HDH could be responsible for the observed activity.

Complete Oxidation Reactions Catalyzed by the Mutant Enzymes. The molar stoichiometry of NADH production with a given quantity of histidinol showed that the complete reaction was carried out for H98N, H326N, H366N, and H418Q. With the least active mutant enzyme, H261N, the use of a molar excess of enzyme over substrate showed 1.9 mol of NADH·mol of histidinol^{-1} (Table 2), indicating that histidinol was completely oxidized. With a substoichiometric quantity of enzyme, the apparent stoichiometry of NADH production ranged from 1.3 to 1.7. Thin-layer chromatography showed formation of [^{14}C]histidine after WT, H261N, and H326N were incubated with [^{14}C]histidinol and NAD (results not shown), confirming the ability of H261N and H326N to catalyze the complete HDH reaction.

Kinetic Isotope Effects of Mutant Enzymes. In WT, the second hydride transfer step contributes to rate limitation (*34*, *64*). To determine whether the mutant enzymes showed a rate-limiting hydride transfer step, KIE were determined for WT and mutant enzymes with (H,H) and (D,D)histidinol (Table 2). WT showed a 2.4-fold ^DV deuterium KIE with (D,D)histidinol, similar to the reported values by Grubmeyer and Teng (*34*). H98N, H366N, and H418Q showed ^DV values between 2.0 and 2.5, indicating that hydride transfers were at least partially rate limiting in these three mutants. For H326N, the results were strikingly different, with $^DV = 1.0$ and $^D(V/K) = 0.86$. In the case of H261N, a ^DV effect of 2.0 was observed with $^D(V/K) = 1.5$. With (H,D)histidinol [this preparation contained 58% (H,D)histidinol and 42% (D,D)-histidinol], WT showed a ^DV deuterium KIE of 2.2, whereas H261N showed a ^DV of 1.5.

Substrate Rescue Experiments with Mutant Enzymes. Results of KIE indicated that, with H261N, the first, rather than the second, hydride transfer was more rate limiting when compared to WT. To examine the state of the remaining unoxidized substrate during catalysis by H261N and H326N, experiments were performed in which a small quantity of WT was added to rescue any remaining free histidinol during its oxidation by a superstoichiometric quantity of mutant enzyme. Upon addition of WT to the slow reaction catalyzed by H261N, all histidinol was immediately oxidized at a rate similar to WT alone, demonstrating that remaining substrate-reducing equivalents were readily accessible to WT. In contrast, when a substoichiometric amount of histidinol was added to H326N to initiate catalysis, subsequent addition of WT did not affect the rate of NADH production (data not shown).

Binding of Histidinol to WT and H261N. Equilibrium binding of [^{14}C]histidinol to WT indicated a K_D for histidinol of 12.2 ± 1.9 μM and with 1.4 binding sites/enzyme dimer, similar to published values (*51*). With H261N, the K_D for [^{14}C]histidinol was 197 ± 25 μM, with 2.0 binding sites/enzyme dimer.

Kinetics with Imidazolyl Propanediol. Imidazolyl propanediol is a poor alternative substrate for WT (*53*). Under standard assay conditions, WT showed 140-fold decrease in k_{cat} for imidazolyl propanediol compared to histidinol, with K_M values for imidazolyl propanediol and NAD increased by 24- and 7-fold, respectively (Table 3). H98N showed a similar decrease in k_{cat} with this alternative substrate. H418Q

Table 3: Kinetics with Imidazolyl Propanediol

	K_M		
enzyme	imidazolyl propanediol (μM)	NAD (mM)	k_{cat} (s^{-1})
WT	240 ± 30	7.3 ± 1.4	0.08
H98N	nd[a]	nd	0.04
H261N	nd	nd	undetectable
H326N	26 ± 3	0.7 ± 0.1	0.003
H366N	900 ± 200	0.9 ± 0.2	0.0027
H418Q	450 ± 30	14.0 ± 3.5	0.001

[a] nd, not determined.

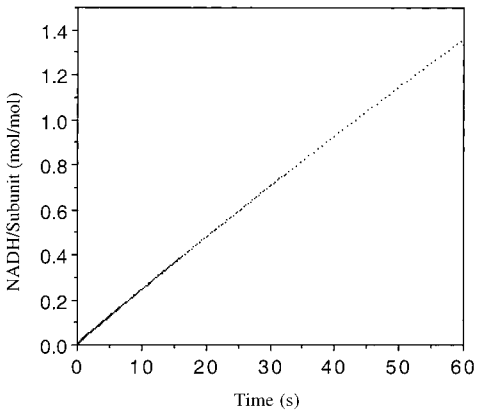


FIGURE 1: Stopped-flow experiment with H326N. Enzyme (192 μM subunit) was preincubated with 4 mM (H,H)histidinol in one syringe and mixed with an equal volume from another syringe containing 20 mM NAD, both solutions were temperature equilibrated at 30 °C. Buffer used contained 50 mM sodium glycine (pH 9.0) and 0.5 mM $MnCl_2$. Results show NADH produced per enzyme subunit against time in seconds.

exhibited an approximate 600-fold decrease in k_{cat}, and a 28-fold increase in K_M for imidazolyl propanediol compared to histidinol, whereas its K_M value for NAD was unchanged. H261N, whose activity was close to the limit of detection even with histidinol, did not show detectable activity with this alternative substrate. With H366N, k_{cat} for imidazolyl propanediol was 1800-fold lower than the rate with histidinol, the K_M for imidazolyl propanediol increased by 8-fold compared to histidinol, and K_M for NAD was increased by approximately 5-fold. H326N showed an approximate 8-fold decrease in k_{cat} with imidazolyl propanediol, a 15-fold increase in K_M for this alternative substrate when compared to histidinol, and with no effect on the K_M for NAD.

Pre-Steady-State Kinetics. Stopped-flow results showed that the initial rate of NADH production with H326N was linear, with a steady-state rate of 0.023 mol of NADH·mol of subunit^{-1} (corresponding to a k_{cat} of 0.012 s^{-1}), with no evidence for a burst phase (Figure 1).

Effects of pH on Catalysis by Mutant Enzyme. Profiles of log(k_{cat}) versus pH for all mutant enzymes were similar to WT, whose single titratable group gives a pK_a of 8.17 [Figure 2 (*34*)]. H261N and H326N showed pK_a values of 7.9 and 7.7, respectively. The log(k_{cat}/K_M) pH profiles of histidinol for WT, H261N, and H326N are shown in Figure 3. Both WT and H326N exhibited a single essential base with pK_a = 8.4 (*34*) and 8.2, respectively. In contrast, H261N showed a linear relationship between log(k_{cat}/K_M) and pH from 6.5 to 9.5 with a slope of 1.0, suggesting that productive association of histidinol with H261N is governed by a base with pK_a above 9.5.

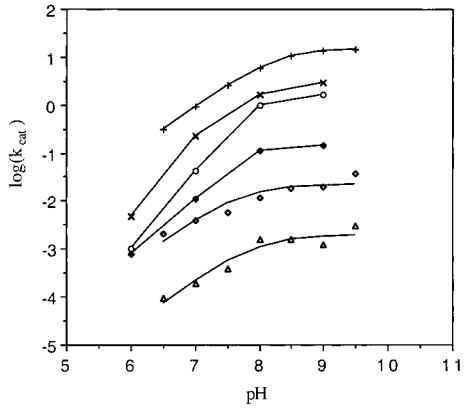


FIGURE 2: pH Dependence of log(k_{cat}) with WT and mutant enzymes. The symbols used are: WT (+), H98N (×), H261N (△), H326N (◇), H366N (○), and H418Q (◆). The unit for k_{cat} is inverse seconds (s^{-1}). With WT, H261N, and H326N, solid lines represent theoretical fittings. With H98N, H366N, and H418Q, solid lines represent interpolations.

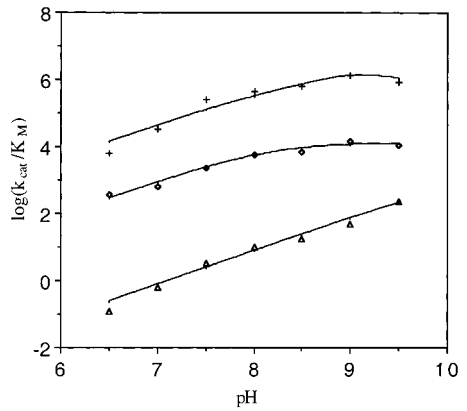


FIGURE 3: pH Dependence of log(k_{cat}/K_M) for histidinol with WT and mutant enzymes. The symbols used are WT (+), H261N (△), and H326N (◇). The unit for k_{cat}/K_M is inverse molarity inverse seconds ($M^{-1}\cdot s^{-1}$). The solid lines represent theoretical fittings. With H261N statistical analysis yielded a pK_a value of 10.28 ± 0.49.

Roles of Histidine Residues in Metal Ion Ligation. HDH contains a single essential Zn^{2+} per subunit. When stimulatory Mn^{2+} is added, this Mn^{2+} binds, probably by exchange, to occupy a Zn^{2+} site (*39*). Mutation of metal-binding histidine residues in metalloenzymes has been shown to result in altered responses to chelator and decreased affinities for metal ions (*40*, *65*−*69*). With HDH, we observed that H418Q was inactivated by the presence of EDTA in the assay medium, a treatment which did not affect the activities of WT and other mutant enzymes. Since two of the mutant enzymes (H261N and H326N) showed very low activity, it was possible that, as isolated, those mutant enzymes were depleted of divalent metal ion and unable to bind it. To test this hypothesis each enzyme was treated with 1,10-phenanthroline and EDTA, a treatment which was shown to inactivate WT (*39*). Similar to WT, we observed that this treatment also inactivated all mutant enzymes. Incubation of the inactivated enzyme in the presence of 1 mM $MnCl_2$, after removal of 1,10-phenanthroline and EDTA, reactivated the enzymes to 80−110% of their original activities (data not shown).

To further understand the effects of the mutations on metal ion affinity, metal ion contents for WT and mutant enzymes

Table 4: Metal Content of WT and Mutant HDH[a]

	(g atom of/mol of dimer)			
	storage		assay	
enzyme	Zn^{2+}	Mn^{2+}	Zn^{2+}	Mn^{2+}
WT	1.4	0.0	0.8	0.8
H98N	1.2	0.2	0.2	0.6
H261N	0.2	0.4	0.0	0.0
H326N	0.8	0.2	0.2	0.8
H366N	1.6	0.2	0.8	1.0
H418Q	0.6	0.2	0.0	0.0

[a] Crystalline enzyme or glycerol samples were dissolved in 20 mM Tris-succinate (pH 6.0) supplemented with 0.1 mM $MnCl_2$ (storage) or 50 mM sodium glycine (pH 9.0) supplemented with 0.5 mM $ZnCl_2$ (assay). Dissolved enzyme samples were loaded onto centrifuge columns (*50*) preequilibrated with either 20 mM Tris-succinate (pH 6.0) or 50 mM sodium glycine (pH 9.0). To the eluates were added 0.1 mM $MnCl_2$ (for samples at pH 6.0) or 0.5 mM $MnCl_2$ (for samples at pH 9.0). After incubating at 30 °C for 5 min, samples were loaded onto centrifuge columns preequilibrated with 0.5 M sodium Hepes (pH 7.0) to remove free metal ion. Eluates were then used in determining metal contents (see Materials and Methods).

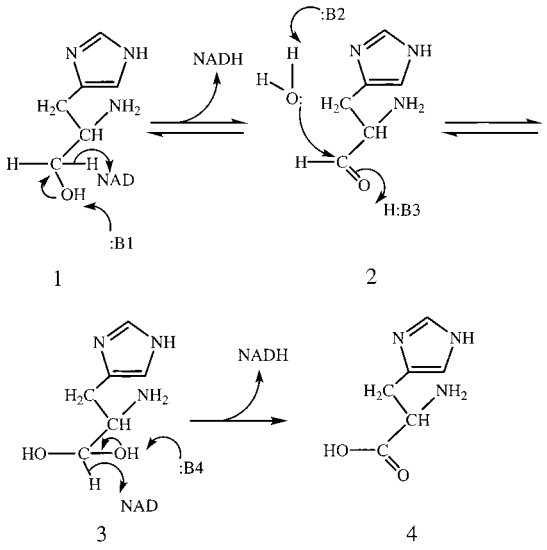
under "storage" (pH 6.0) and "assay" (pH 9.0) conditions (see the Materials and Methods) were determined after removal of free metal ions by centrifuge columns (*50*). Zinc contents for WT, H98N, and H366N were similar under storage conditions (1.2−1.6 g atom of Zn^{2+}/mol of dimer, Table 4). Reduced zinc content was seen with H326N (0.8 g atom of Zn^{2+}/mol of dimer), H418Q (0.6 g atom of Zn^{2+}/mol of dimer), and H261N (0.2 g atom of Zn^{2+}/mol of dimer). EPR spectroscopy showed negligible manganese ion contents for all enzymes under storage conditions. Under assay conditions, WT and H366N showed 0.8 g atom of Zn^{2+}/mol of dimer, and 0.8−1.0 g atom of Mn^{2+}/mol of dimer. For H98N and H326N, zinc ion contents were decreased to 0.2 g atom of Zn^{2+}/mol of dimer, and manganese ion contents were determined to be 0.6 and 0.8 g atom of Mn^{2+}/mol of dimer, respectively. The most dramatic losses of metal ions were observed in H261N and H418Q, which did not contain detectable Zn^{2+} or Mn^{2+} following centrifuge column chromatography under assay conditions.

Incubation of WT, H261N, H326N, and H418Q in Tris-succinate buffer (pH 6.0) supplemented with 0.5 mM $ZnCl_2$ showed 1.6, 1.4, 1.4, and 1.0 g atom of Zn^{2+}/mol of dimer, respectively. After a second round of centrifugation chromatography, the zinc ion contents for H261N and H418Q were reduced to 0.4 g atom of Zn^{2+}/mol of dimer. WT and H326N showed no significant loss of metal ion following the same treatment.

DISCUSSION

We have mutated the five conserved histidine residues (His-98, His-261, His-326, His-366, and His-418) in *S. typhimurium* HDH to either asparagine or glutamine to test the hypothesis that these residues might serve as the active base or zinc ligands. Scheme 1 shows a proposed mechanism for HDH identifying potential sites of acid-base catalysis. Enzymic bases are required in (1) removal of the hydroxyl proton before or during hydride transfer from histidinol during its oxidation (B1), (2) the deprotonation of attacking water (B2), (3) protonation of histidinal hydrate (*gem*-diol)-(H:B3), and (4) the deprotonation of histidinal hydrate by B4 during oxidation to form histidine. Although the model

Scheme 1: Proton Transfers during Catalysis by HDH[a]



[a] 1. Proton transfer by base B1 and hydride transfer from histidinol during the histidinol oxidation half-reaction, resulting in histidinal. 2. Activation of water by base B2. Base B3-catalyzed proton transfer to histidinal carboxyl oxygen to form the hydrate. 3. Proton transfer from histidinal hydrate to base B4 and hydride transfer during the histidinal oxidation half-reaction. 4. Histidine formation.

uses an aldehyde hydrate as an intermediate, other chemistries of aldehyde activation would require similar use of base to deprotonate attacking nucleophile. The roles of B1, B3, and B4 could be fulfilled by a single residue. Our results showed that His-261, either directly or via a metal-bound water, acts as B1, whereas His-326 appears to act as B2. We first discuss results with H261N and H326N, then the other three mutant enzymes.

His-261 Is Involved in Histidinol Deprotonation and Is a Metal Ligand. Nagai and Ohta (*40*) presented mutagenesis studies on the analogous His-261 of the *Brassica oleracea* HDH with results broadly similar to ours. It was reported that *Brassica* H261N did not contain Zn^{2+} or Mn^{2+} and did not catalyze detectable oxidation of histidinol. In the presence of exogenous zinc or manganese ion, the mutant *Brassica* enzyme catalyzed qualitatively detectable histidinal reduction, and histidinal oxidation occurred at a rate about 5% of WT. These observations led the authors to propose that His-261 in *Brassica* HDH is a ligand to a catalytic zinc ion.

Mutation of His-261 in the *S. typhimurium* HDH caused an alteration of enzyme properties so extreme that results are somewhat difficult to interpret. The rate of overall histidinol oxidation suffered a 7000-fold decrease, such that 10 min is required for a single enzyme turnover (Table 2). A similar reduction in catalytic activity might have occurred with the H261N *B. oleracea* HDH with the residual activity not detected. Although these enzymes were expressed in a *hisD* deletant, which eliminates host enzyme contamination (*70*, *71*), mistranslation (*72*) can occur even with the infrequently mistranslated AAC codon (*55*, *73*) and the *rpsL* mutation (*72*). Therefore, it is conceivable that the low remaining activity observed in H261N came from a minute population of enzymatically active contaminant that arose from mistranslation. The stoichiometric binding of [^{14}C]-histidinol to free H261N and the alterations of KIEs (Table 2) and metal-binding (Table 4) properties argued against this possibility.

Using the centrifuge column method (*50*), we were unable to detect enzyme-bound zinc or manganese ion with H261N under assay conditions (Table 4). Nevertheless, demetalation followed by assay in metal-free buffer resulted in complete loss of activity, which was restored when exogenous metal ion was added, indicating that enzyme-bound metal ion was essential for the low activity observed. We also observed that incubation of H261N in $ZnCl_2$-containing buffer at pH 6.0 resulted in significant zinc ion binding, demonstrating that the mutation did not totally abolish metal ligation ability. The loss of bound zinc ion upon an additional round of centrifugation chromatography suggested that the mutation decreased the ability of the mutant enzyme to retain bound metal ion. In separate experiments, we also established that increasing the manganese ion concentration in assay medium produced no significant increase in enzyme activity with H261N (data not shown). It is likely that H261N exists under assay conditions as labile E·Mn^{2+} or E·Zn^{2+} complexes, whose rapid dissociation rates allow loss of metal ion during centrifuge column separations. Thus, the low enzymatic activity is that of the enzyme·metal ion complex.

We used KIE to determine the effect of the mutation on the individual half-reactions. In the case of WT at pH 9, the second hydride transfer step contributes significantly to the observed ^{D}V isotope effect (*34*, *64*). With H261N, the ^{D}V effect with (H,D)histidinol was 1.5, significantly lower than the ^{D}V effect with (D,D)histidinol ($^{D}V = 2.0$; Table 2) and suggesting that the first hydride transfer is partially rate determining in H261N. Results of the substrate rescue experiment showed that bound histidinol dissociated readily from its complex with H261N when compared to WT, in support of the hypothesis that the histidinol oxidation rate in H261N has been specifically reduced and directly contributes to rate limitation. The 16-fold increase in the K_D for histidinol with H261N when compared to WT is consistent with a reduced histidinol oxidation rate.

If the decreased activity of H261N were a result of replacement of an enzymic base, the pH profile of k_{cat} and k_{cat}/K_M might be altered. In WT, a single titratable group with $pK_a = 7.9-8.4$ acts as general base during catalysis (*34*). For H261N, the log(k_{cat}) pH profile showed a titratable group with pK_a similar to that of WT (Figure 2). In contrast, the log(k_{cat}/K_M) pH profile with H261N gave a slope of 1.0, and did not show a pK_a value within the pH range tested (Figure 3), strongly indicating that productive association of histidinol with H261N was dependent on a group with pK_a above 9.5. A likely candidate for the replacement group would be solvent or metal-coordinated water.

Nagai and Ohta (*40*) proposed that His-261 in *Brassica* HDH acts as a ligand to the zinc ion, which serves to polarize histidinol and aid its deprotonation. The proposed catalytic role for the bound zinc ion in *Brassica* HDH is similar to that proposed for horse liver ADH (*74*, *75*). Nuclear magnetic resonance studies conducted with $^{113}Cd^{2+}$-substituted *Brassica* HDH were interpreted by Kanaori et al. (*41*, *76*) to indicate that the hydroxyl group of histidinol interacts with the metal ion. A similar role for His-261 in *Salmonella* HDH can explain the shift of the enzymic base in the k_{cat}/K_M versus pH profile. With the substitution of His-261 by asparagine, the metal-binding properties of the enzyme are altered, and the pK_a of metal-bound water molecule may be dramatically increased, lowering the concentration of the catalytically active enzymeH^+·histidinol·NAD ternary complex.

Role of His-326 in Catalysis. Like H261N, H326N showed a severely depressed k_{cat} (Table 2). Results from the substrate rescue experiments showed that the mutant enzyme could bind substrate and catalyze the HDH reaction, and ruled out the possibility that the observed enzymatic activity came from a minute population of contaminant. The lack of any detectable substrate deuterium KIE (Table 2) suggested that a step in the reaction sequence other than hydride transfer had become rate limiting. This might represent a proton transfer normally catalyzed by an enzymic base.

The lack of a $^{D}V/K$ isotope effect (Table 2) and the results from substrate rescue experiments showed that histidinol in the H326N·histidinol·NAD ternary complex was highly committed. Surprisingly, stopped-flow studies did not show an initial burst of NADH production with H326N (Figure 1), indicating that the mutation affected the first half-reaction. The titratable group in both log(k_{cat}) and log(k_{cat}/K_M) pH profiles (Figures 2 and 3, respectively) showed that even though the mutation had reduced k_{cat} by 500-fold, the mutation did not remove the essential enzymic base observed in WT profiles. A likely role for His-326 is that this residue normally serves to deprotonate the attacking nucleophile (H_2O in Scheme 1). The absence of a pre-steady-state burst indicates that hydrate formation is closely associated with the first hydride transfer. With human liver mitochondrial aldehyde dehydrogenase, substitution of Glu-268 by aspartate resulted in the lack of a pre-steady-state NADH burst, unlike the wild-type enzyme. The results were interpreted in indicating a role for Glu-268, acting as an enzymic base, to deprotonate the attacking nucleophile (*20*).

The 8-fold decrease in k_{cat} with imidazolyl propanediol when compared to histidinol was unexpectedly small (Table 3). Nonetheless, H326N still catalyzed the oxidation of this alternative substrate about 30-fold slower than WT. It was observed that H326N did not show substrate ^{D}V KIE with dideuterated imidazolyl propanediol (data not shown), suggesting that the impaired proton transfer resulting from the H326N mutation contributed substantially to the overall rate limitation with this alternative substrate.

Kanaori et al. (*41*) suggest that the homologous His-327 in *Brassica* HDH is a metal ligand. Although metal ion content measurements (Table 4) showed decreased metal ion contents with H326N, incubation in buffer containing zinc ion showed substantial zinc binding, which was not reduced by additional centrifugation chromatography (unlike H261N), arguing against His-326 in *Salmonella* HDH acting as a metal ion ligand. Thus, the current data support a role for His-326 as an enzymic base rather than a metal ion ligand.

Roles of His-366 and His-98. Nagai and Ohta (*40*) stated that substitutions of His-94 and His-367 in *Brassica* HDH (analogous to His-98 and His-366 in *Salmonella* HDH) did not affect catalytic activities. We observed that H366N behaved similarly to WT in specific activity, chelator sensitivity, stoichiometry of NADH produced:histidinol added, pH profile, deuterium KIE, and metal ion affinities. It did, however, show a readily measured increase in the K_M for histidinol and imidazole propanediol (about 8-fold in both cases; Tables 2 and 3), whereas the K_M for NAD was unaffected, suggesting a role for His-366 in interaction with histidinol.

Substitution of His-98 by asparagine did not show substantial differences from WT in experiments described, and any catalytic role was therefore precluded. The H98N did show altered Zn^{2+} content under assay conditions, but it is not clear whether this results from any direct role as a metal ligand.

His-418 Involvement in Metal Ligation. The sensitivity to the presence of chelator EDTA showed that substitution of His-418 by a glutamine caused perturbation in the metal ion environment. Metal ion content measurements showed that H418Q bound substantially less metal ion when compared to WT (Table 4). Furthermore, we observed zinc ion binding when H418Q was preincubated with $ZnCl_2$-containing buffer at pH 6.0, and the bound zinc ion was lost upon additional centrifugation chromatography, indicating that the H418Q mutation weakened the ability to retain bound metal ion, consistent with a role for His-418 as metal ligand. A 14-fold increase in K_M for NAD observed with H418Q suggested a role for His-418 in interaction with coenzyme; however, this mutant enzyme showed extreme thermolability, and the increased K_M for NAD could be a result of structural perturbation. Substitution of a zinc ligand in recombinant rat sorbitol dehydrogenase also resulted in increased K_M for NAD (15-fold), which was attributed to perturbation of the active site (*77*). It is interesting that although both His-261 and His-418 interact with bound Zn^{2+}, the results of their mutation differ dramatically. H418Q retained about 4% of WT activity, but it loses metal ion rapidly, becoming inactive. H261N, in contrast, shows little direct chelator sensitivity, but the metal-containing enzyme itself has extremely poor activity. Substitutions of catalytic zinc ion ligands by asparagine and glutamine have been shown to affect metal ion binding, activities, and/or thermostability in thermophilic bacteria *Thermoanaerobacter brockii* ADH, carbonic anhydrase II, *E. coli* alkaline phosphatase, and arginase (*66*−*69*, *78*).

Proposed Mechanism of HDH Catalysis. In this work, we have presented data in support of enzymic base roles for His-261 and His-326 and metal ion ligation roles for His-261 and His-418. We also proposed that the zinc ion in *Salmonella* HDH is catalytic, in agreement with work of Nagai and Ohta (*40*) and Kanaori et al. (*41*). The proposed mechanism of horse liver ADH-catalyzed oxidation of benzaldehyde, which involves an aldehyde hydrate monoanion (*3*), was adapted to explain the second half-reaction catalyzed by HDH. Adaptation of Scheme 1 would then require the B1 to be a zinc-bound hydroxide ion, which catalyzes proton transfer from the hydroxide of histidinol to form a histidinol alkoxide ion. The histidinol alkoxide ion then displaces the zinc-bound water (with the alkoxide ion liganded to the catalytic zinc ion). His-326 (B2) catalyzes proton transfer from a water molecule, forming a hydroxide ion, which undergoes nucleophilic attack at the carbonyl carbon, and results in histidinal hydrate monoanion. (On the basis of the stopped-flow results with H326N, the nucleophilic attack must be closely associated with the hydride transfer to first NAD.) After second hydride transfer to NAD, histidine is formed. In the proposed mechanism, the roles of His-261, acting as a Zn^{2+} ligand, and of His-326, acting as base B2 (deprotonating water), render them essential for catalysis. Although His-418 is also another Zn^{2+} ligand, a direct role in catalysis cannot be established. It should be noted that the proposed mechanism is different in many respects from the other two four-electron oxidoreductases: HMGR (*26*) and uridine diphospho-α-D-glucose dehydrogenase (*79*, *80*), which both use hemiacetals (rather than aldehyde hydrate) during the aldehyde oxidation half-raction, are not zinc metalloenzymes, and do not show sequence similarity to HDH (*79*). Instead, the proposed mechanism is similar to horse liver ADH and suggests functional convergence within the dehydrogenases.

ACKNOWLEDGMENT

We thank Dr. David E. Ash and Mr. Thomas Sossong for their technical guidance in the EPR experiments and Mr. Ryan Dorfman for synthesis of (H,D)histidinol.

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BI982758P