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Site-Directed Mutagenesis of Histidine-90 in *Escherichia coli* L-Threonine Dehydrogenase Alters Its Substrate Specificity[1]

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***Escherichia coli* L-threonine dehydrogenase is a member of the Zn^{2+}-containing alcohol/polyol dehydrogenase family. Methylation of His-90 of L-threonine dehydrogenase was recently found to cause total inactivation (J. P. Marcus and E. E. Dekker, 1995 *Arch. Biochem. Biophys.* 316, 413–420). Since His-90 is not conserved among the related dehydrogenases, this residue was changed to arginine, asparagine, and alanine by site-directed mutagenesis in order to probe its role. All three purified, homogeneous mutants, like wild-type enzyme, contained one Zn^{2+} atom/subunit and exhibited a sequential catalytic mechanism; the k_{cat} value for each, however, was reduced ~10-fold. The K_m value for threonine was elevated from 3 mM for wild-type enzyme to 31, 328, and 417 mM, respectively, for mutants H90R, H90N, and H90A. The activation energy of catalysis for mutant H90A was increased by 6.6 kcal/mol, suggesting that in the wild-type enzyme His-90 forms at least one crucial hydrogen bond in the transition state. Whereas wild-type enzyme catalyzed the oxidation of threonine amide (0.75 M) about twice as fast as this same concentration of threonine or 0.375 M L-2-amino-3-hydroxypentanoate, the reaction rate of mutant H90A with 0.75 M threonine amide or threonine methyl ester was 33- to 35-fold higher than with this level of threonine. Similarly, mutant H90N used 0.75 M threonine methyl ester or threonine amide as substrate 9- to 13-fold better than it used this concentration of threonine. Mutants H90A and H90N were more reactive with 0.225 M L-threonine hydroxamate than with 0.75 M threonine, but mutant H90A did not oxidize L-2-amino-3-hydroxypentanoate (0.375 M) and mutant H90N used this substrate poorly. The best substrates for mutant H90R were threonine methyl ester, threonine, and threonine amide (all tested at 0.75 M); 0.375 M L-2-amino-3-hydroxypentanoate was a poor substrate. The isolation and characterization of these first His-90 mutants of *E. coli* L-threonine dehydrogenase confirm the importance of this residue in catalysis and suggest that His-90 is an active-site residue which modulates the substrate specificity of L-threonine dehydrogenase.**

Key Words: **site-directed mutagenesis; protein isolation and purification; steady-state kinetics; substrate specificity; enzyme active site.**

L-Threonine dehydrogenase (EC 1.1.1.103) catalyzes the NAD^+-dependent oxidation of threonine to form 2-amino-3-ketobutyrate, which is the first step in the major route for threonine metabolism in prokaryotes (1, 2) and eukaryotes (3). *Escherichia coli* TDH[4] is a homotetrameric enzyme (M_r 149,000) which contains one Zn^{2+} atom/subunit (4, 5) and is a member of the medium-chain, zinc-containing alcohol/polyol dehydrogenase family (6). A progressive alignment of members of this group reveals that the amino acid sequence of *E. coli* TDH is 26–27% homologous with horse LADH E, human liver ADH, or yeast ADH1 as well as with human or sheep liver SDH (7). *E. coli* TDH shows no similarity with the partial amino acid sequence so far obtained for mammalian (porcine) TDH (8). *E. coli*

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[4] Abbreviations used: TDH, L-threonine dehydrogenase; ADH, alcohol dehydrogenase; LADH, horse liver ADH isozyme E; SDH, sorbitol (glucitol) dehydrogenase; MNBS, methyl *p*-nitrobenzenesulfonate; 2-ME, 2-mercaptoethanol.

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TDH, however, is unique among these enzymes in that only its catalytic activity is significantly enhanced by added Mn^{2+} or Cd^{2+} (9, 10).

So far, two amino acid residues crucial for TDH catalysis have been identified. Alkylation of Cys-38 by iodoacetate inactivates TDH and abolishes its ability to bind Mn^{2+} (4, 11). Unexpectedly, selective reaction of Cys-38 with *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K) was also found to inactivate TDH (12). Cys-38 of TDH aligns with Cys-46 of LADH, which is one of three ligands to the catalytic Zn^{2+} atom in this enzyme (6). The other critical residue in TDH is a histidine; incubation of TDH with [^{14}C]methyl *p*-nitrobenzenesulfonate eliminates enzymatic activity, concomitant with incorporation of one methyl group/enzyme subunit (13). Radioactivity was found to be incorporated into 3-methyl-*N*-histidine, suggesting a specific orientation of the side chain of this residue within the tertiary structure of TDH. Isolation and amino acid sequencing of the major radioactive tryptic peptide of methylated, inactivated TDH showed this residue was His-90 (13). Whereas L-threonine and other substrates/substrate analogs provided ~60% maximal protection, NAD^+ had no effect and NADH enhanced the inactivation (13). The basis for this inactivation remains unclear, as no residue analogous to His-90 of TDH is apparent in the other dehydrogenases with which it is homologous.

His-90 in TDH aligns with Thr-94 of LADH (6,7). In LADH, Thr-94 is adjacent to Phe-93, which aromatic amino acid residue forms part of the substrate-binding pocket of this enzyme (14, 15). The similarity of amino acid sequences between TDH and LADH in this region suggested that His-90 of TDH might line its substrate-binding site. To explore such a role, His-90 was changed by site-directed mutagenesis to alanine, asparagine, and arginine. The data obtained with the purified homogeneous mutant enzymes indicate that His-90 does affect the substrate specificity of TDH.

MATERIALS AND METHODS

NADH, L-threonine, and threonine analogs were purchased from Sigma Chemical Co., while $MnCl_2 \cdot 4H_2O$ (99.5% pure) and $CdCl_2 \cdot 2.5\ H_2O$ were products of J. T. Baker Chemical Co. and Aldrich Chemical Co., respectively. NAD^+ was obtained from United States Biochemical Corp. The concentrations of coenzyme solutions were estimated using $\epsilon_{260} = 18{,}000\ M^{-1}\ cm^{-1}$ for NAD^+ and $\epsilon_{340} = 6{,}220\ M^{-1}\ cm^{-1}$ for NADH, whereas those for PCR primer solutions were calculated assuming $A_{260} = 1.0$ for 20 μg of primer/ml. TDH enzymatic activity was measured at 37°C with a Cary 219 spectrophotometer equipped with thermostated cuvette holders by following the increase in absorbance at 340 nm due to NADH formation.

DNA preparations. Plasmids were isolated after alkaline lysis (16), and single-stranded DNA samples for sequencing were purified from bacteriophage M13mp18 by precipitation with polyethylene glycol (17). DNA was analyzed by agarose gel electrophoresis and, when necessary, fragments were extracted from the gels using QIAEX kits (Qiagen, Inc.).

Construction of pTDH (kbl$^-$ tdh$^+$ amp^r). After pDR121Δ (*kbl*$^+$ *tdh*$^+$ *amp*^{r}) (18) was incubated with 1 unit of *Ksp*I for 1 h at 37°C, 1.5 μl of Promega buffer D and 10 units of *Eco*RV were subsequently added and the incubation was continued for an additional hour. Introduction of a dNTP mixture brought the concentration of each dNTP to 0.1 mM before 2 units of T4 DNA polymerase were added and the mixture was incubated at 10°C for 20 min. The blunt-end plasmid so formed was gel-purified and thereafter incubated for 12 h at 16°C with 3 units of T4 DNA ligase. This mixture was used to transform DH5αF′ cells, which were plated on growth medium containing agar and 25 μg of ampicillin/ml.

Primers for PCR. Oligodeoxynucleotide primers were synthesized by the Protein Structure Core Facility at this university. Two outside primers were used in the construction of all three mutants of His-90 (see Table I). Forward and reverse inside primers which contained the changed codons for residue 90 from His (5′-CAT-3′) to Ala (5′-GCC-3′), Asn (5′-AAT-3′), or Arg (5′-CGT-3′) were also prepared.

Construction of the mutant cassettes by PCR. A recombinant PCR method was used (19). Reactions (0.1 ml) were carried out in 0.5-ml PCR tubes using a Perkin–Elmer DNA thermal cycler 480. Mixtures containing Vent$_R$DNA polymerase buffer (1×), 2 mM $MgSO_4$, 200 μM each dNTP, 0.5 μM each primer, and ~0.25 ng of pDR121Δ were used to synthesize the left and right fragments. Just before the thermal cycling program was started (30 cycles; 90 s at 94°C, 60 s at 55°C, and 45 s at 72°C), 1 unit of Vent$_R$DNA polymerase was added under a layer of mineral oil. Products were purified with QIAquick spin columns (Qiagen, Inc.). The recombinant PCR mixtures contained *Taq* DNA polymerase buffer (1×), 2.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM of each the outside primers, together with 1 μl of each of the left and right PCR fragments. *Taq* DNA polymerase (2.5 units) was added just before thermal cycling was begun (30 cycles; 60 s at 94°C, 60 s at 50°C, and 45 s at 72°C). The products were purified using spin columns. These PCR fragments as well as pTDH were cleaved by incubating them with *Ava*I, and the resultant mutant cassettes and the linearized vector were gel purified. After each cassette was ligated into pTDH, DH5αF′ cells were transformed with the ligation mixtures, then plated on growth medium containing agar and 50 μg of ampicillin/ml and finally grown at 37°C.

Sequencing of mutant DNA in M13mp18. The dideoxynucleotide chain termination method (17) was followed using Sequenase Version 2.0 (United States Biochemical Corp.) and [α-^{35}S]dATP (Amersham Corp.). The *Eco*RI/*Sma*I fragment of pTDH mutants was subcloned into M13mp18 replicative form DNA, and the complete DNA sequences of both the *Ava*I mutant cassettes and the flanking regions were determined.

Purification of TDH mutants H90A, H90N, and H90R. A *Tdh*$^-$ strain of *E. coli*, namely SP1192 (*tdh::cat1212 glyA*$^-$ Δ*recA srl::Tn10*) (18), was transformed with each mutant pTDH, and the resultant mutant strains were used. The procedure of Craig and Dekker (9) was followed to purify TDH. Each mutant protein was analyzed for purity by SDS–PAGE, and the homogeneous enzymes were stored under argon at 4°C until used. The quaternary structure of the purified mutant enzymes, as well as wild-type TDH and several reference standards of known M_r, was analyzed by nondenaturing 7% PAGE carried out at pH 8.3 using a Tris/glycine buffer system (20).

Determination of the zinc content of wild-type TDH and the mutant forms. Samples of wild-type TDH and the mutants were dialyzed for 24 h at 10°C against four 1-L changes of 1 mM sodium phosphate buffer (pH 7.0); ~3 mg of such enzyme samples were frozen until submitted for analysis. The level of zinc in each sample and the dialysate buffer was estimated by atomic absorption spectrophotometry (Galbraith Labs, Knoxville, TN).

CD spectra of wild-type TDH and the mutants. The CD spectra of wild-type TDH and the three mutants were recorded at 25°C using a JASCO J-710 spectropolarimeter in the Protein Structure Core Facility at this university. Samples were scanned at a speed of 200

TABLE I

Primers Used for Construction of Site-Directed Mutants of *E. coli* L-Threonine Dehydrogenase

Primer	Sequence
Left, outside	5′-TTATCGCCTGAGGAATTCAGATGAAAGCG-3′
Right, outside	5′-CACCAGATATTCGGC-3′
Ala, left	5′-GGTGATGGCGCCTTCGCCAGAAACGCG-3′
Ala, right	5′-GGCGAAGGCGCCATCACCTGTGG-3′
Asn, left	5′-GGTGATATTGCCTTCGCCAGAAACGCG-3′
Asn, right	5′-CGGCGATCGCGTTTCTGGCGAAGGCAATATCACCTGTGG-3′
Arg, left	5′-AATGACCACAGGTGATACGGCCTTCGCCAG-3′
Arg, right	5′-GTTTCTGGCGAAGGCCGTATCACCTGTGGTC-3′

Note. Mismatched bases are underlined. The mismatches in the left, outside primer (which was designed for and used in unrelated experiments) were not incorporated in the final His-90 mutant cassettes prepared in the studies described in this report.

nm/min with a response time of 0.5 s; the path length of the sample cell was 0.1 cm.

Heat stability of wild-type TDH and the mutant forms. Wild-type TDH and the mutants (1 mg/ml in 0.1 M sodium phosphate buffer, pH 8.0) were incubated for 40 min at 60°C in PCR tubes. Residual enzymatic activity was measured at 37°C in mixtures containing 0.2 M Tris–HCl buffer (pH 8.4), 15 mM NAD^+, and 0.75 M L-threonine.

pH-activity profiles. The rate of the reaction catalyzed by wild-type TDH and the mutants was measured at 25°C in reaction mixtures which contained 10 mM NAD^+, 0.6 M L-threonine, and 0.2 M sodium phosphate (pH 6.3–8.1), Tris–HCl (pH 7.8–9.0), 2-(*N*-cyclohexylamino)ethanesulfonic acid–NaOH (pH 8.6–10.4), or 3-(cyclohexylamino)-1-propanesulfonic acid–NaOH (pH 9.7–11.4) buffer.

Effect of added Cd^{2+} or Mn^{2+} on the activity of wild-type TDH and the three mutants. The level of enzymatic activity of wild-type TDH and the His-90 mutants was measured at 37°C after incubation of the enzyme on ice for 15 min in 50 mM Tris–HCl buffer (pH 8.4) in the absence of any added metal ion or with either 1 mM $MnCl_2$ plus 5 mM 2-mercaptoethanol or 70 μM $CdCl_2$ (8, 9). Controls were assayed after incubating the enzyme with 1 mM $MnCl_2$ or 5 mM 2-ME. Assay mixtures contained 0.2 M Tris–HCl buffer (pH 8.4), 10 mM NAD^+, and 0.78 M L-threonine.

Inactivation of Cd^{2+}-stimulated and native TDH by MNBS. Native TDH as well as enzyme which had been stimulated by 70 μM $CdCl_2$ (5 min at 0°C) were reacted with 2 mM MNBS, and the level of enzymatic activity remaining after varying times of incubation up to 45 min was assessed as previously described (13).

Steady-state kinetic analyses. In these studies, assays were performed at 37°C in 0.2 M Tris–HCl buffer (pH 8.4); the NAD^+ concentration was varied from 0.9 to 15 mM. For mutant H90R, 20 to 320 mM L-threonine was used, whereas for the H90N and H90A mutants the level of this substrate ranged from 46 to 730 mM. The results obtained were analyzed using the KinetAsyst computer program (IntelliKinetics, State College, PA).

Calculation of $\Delta\Delta G^{\ddagger}$ between wild-type TDH and mutant H90A. The difference in the transition state energy for catalysis between wild-type TDH and mutant H90A ($\Delta\Delta G^{\ddagger}$) was calculated from their kinetic constants using an equation modified for use with a two-substrate enzyme when changes in K_m values are observed for both substrates (21):

$$\Delta\Delta G^{\ddagger} = RT \ln \left[\frac{\left(\frac{k_{cat}}{K_{m,A}\,K_{m,B}}\right)\text{mutant}}{\left(\frac{k_{cat}}{K_{m,A}\,K_{m,B}}\right)\text{wild-type}} \right],$$

where R is the gas constant, T is the absolute temperature, k_{cat} is the turnover number, and $K_{m,A}$ and $K_{m,B}$ are the Michaelis constants, respectively, for NAD^+ and L-threonine.

Determination of the K_i of NADH for wild-type TDH and mutant H90R. The inhibition of threonine turnover by NADH was measured for wild-type TDH and mutant H90R. Assays were performed at 25°C in 0.1 M Tris–HCl buffer (pH 8.4) in the presence of 10 mM (for wild-type TDH) or 250 mM (for mutant H90R) L-threonine. At each concentration of NADH used (0, 50, 100, and 150 μM), the level of NAD^+ was varied from 0.1 to 1.0 mM. KinetAsyst was used to determine the type of inhibition and the K_i values.

Determination of the substrate reactivity of the His-90 mutants. The rate of reaction catalyzed by the mutant enzymes with 0.75 M L-threonine, L-threonine methyl ester, L-threonine amide, with DL-2-amino-3-hydroxypentanoate (0.375 M L-isomer), or with 0.45 M DL-threonine hydroxamate as substrate (0.225 M L-isomer) was determined at 37°C in reaction mixtures containing 0.2 M Tris–HCl buffer (pH 8.4) and 15 mM NAD^+.

RESULTS

Construction of pTDH for use in cassette mutagenesis. The *Ava*I site at position 654 of pDR121Δ was removed to leave two *Ava*I sites in pTDH. The His-90 mutant cassettes were synthesized by PCR and then subcloned into the *Ava*I cassette region of pTDH. The orientation of the cassettes was unambiguous since the *Ava*I sites in pTDH differ (5′-CTCGGG-3′ at nucleotide 1111 and 5′-CCCGGG-3′ at position 1395). The loss of an *Xcm*I site at position 1318 of pTDH in all three mutant constructs as well as incorporation of a unique *Nar*I site at position 1310 of pTDH-H90A was confirmed.

Purification of the mutant forms of TDH. The His-90 mutant enzymes expressed in *E. coli* SP1192 cells were isolated and purified from crude cell extracts by the same procedure used for wild-type TDH. Each mutant enzyme was sequentially adsorbed to and eluted from anion-exchange (diethylaminoethyl-Sephadex) and coenzyme-affinity (Reactive Blue 2-Sepharose CL-6B) resins and judged to be >98% pure by SDS–PAGE (not shown). The yields of pure protein (mg) per 20 g of wet cell paste were H90R, 32; H90N, 78; and H90A, 34.

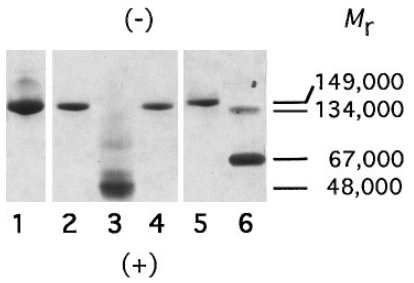

FIG. 1. Nondenaturing PAGE analysis of wild-type TDH and the His-90 mutants. Samples applied (15 μl of a 1 mg/ml solution of each protein) were (M_r): lane 1, wild-type TDH (149,000); 2, mutant H90A; 3, egg albumin (48,000); 4, mutant H90N; 5, mutant H90R; and 6, monomeric (bottom; 67,000) and dimeric (top; 134,000) forms of bovine serum albumin. Migration was from top (−) to bottom (+) and the running buffer was pH 8.3.

Quaternary structure of the mutant enzymes. Wild-type *E. coli* TDH has previously been shown by several methods to be a homotetrameric protein (2, 13). When subjected to nondenaturing PAGE carried out at pH 8.3, all three His-90 mutants were also judged to be homotetrameric proteins as indicated by their migration rates relative to wild-type TDH and reference proteins of known size (see Fig. 1). Mutants H90A (lane 2) and H90N (lane 4) migrated the same as wild-type TDH (lane 1), whereas mutant H90R (lane 5) moved a little more slowly in this electrophoretic system due to the positive charge on Arg-90. While migration in nondenaturing PAGE can be affected by a protein's mass as well as its charge, the regression analysis (Fig. 2) of the standard proteins used in Fig. 1 indicates that a linear relationship exists between the relative migration of the protein and the logarithm of the molecular mass for egg albumin, the monomeric and dimeric forms of bovine serum albumin, and wild-type TDH.

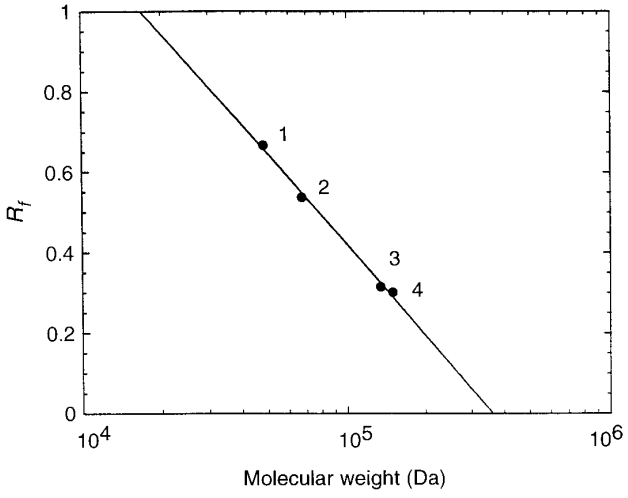

FIG. 2. Regression analysis of the standard proteins shown in Fig. 1. The relative migrations (R_f) of egg albumin (1), monomeric (2) and dimeric (3) forms of bovine serum albumin, and wild-type *E. coli* TDH (4) were determined.

TABLE II

Effect of Added Cd^{2+} or Mn^{2+} on the Enzymatic Activity of Wild-Type *E. coli* TDH and the His-90 Site-Directed Mutants

Addition	Relative velocity (%) for residue at position 90			
	His (wild-type)	Arg	Asn	Ala
None	100	100	100	100
70 μM Cd^{2+}	783	59	71	73
1 mM Mn^{2+}	783	96	31	127
1 mM Mn^{2+} + 5 mM 2-mercaptoethanol	1468	29	5	0
5 mM 2-mercaptoethanol	117	118	65	82

Note. Assays were carried out at 37°C in 0.2 M Tris–HCl buffer (pH 8.4) containing 10 mM NAD^+ and 0.78 M L-threonine. The velocity for no addition was taken to be 100% for each enzyme. The data shown are the averages of two assays, and differences between duplicates were <10%.

Zinc content of wild-type and mutant forms of TDH. The levels of Zn^{2+} present in wild-type TDH and the H90R, H90N, and H90A mutants, as determined by atomic absorption spectroscopy, were respectively 1.2, 1.2, 1.0, and 0.9 mol of Zn^{2+}/mol of enzyme subunit. These values are close to that of 1 mol of Zn^{2+}/mol of enzyme subunit previously reported for wild-type TDH (4). The dialysate buffers were found to contain < 0.3 μM Zn^{2+}, while the concentrations in the enzyme samples (54 μM enzyme subunit) were 47–66 μM.

Secondary structural analysis. The CD spectra (not shown) of wild-type TDH and all three His-90 mutants were nearly identical and displayed similar negative ellipticity at 208 and 220 nm.

Stability of the mutants and wild-type TDH to heat. After wild-type TDH was heated for 40 min at 60°C, it retained 64% of its initial enzymatic activity. Of the mutants, on the other hand, H90R showed 84%, but H90A and H90N only 47 and 24%, respectively, of their initial activity after such treatment.

pH-activity profiles. A pH optimum of 10.5, close to the value of 10.3 previously reported for wild-type TDH (2), was observed for the oxidation of threonine by wild-type TDH as well as by all three mutants (not shown).

Effect of added Cd^{2+} and Mn^{2+} on the activity of wild-type and mutant forms of TDH. Whereas wild-type TDH showed an 8- to 15-fold stimulation of activity after being incubated with added Cd^{2+} or Mn^{2+} plus 2-mercaptoethanol, all three mutants exhibited lower activity after such treatment (Table II). When Cd^{2+} was

TABLE III

Steady-State Kinetic Parameters of Wild-Type *E. coli* TDH and the Three His-90 Mutants

Kinetic constant	Residue at position 90			
	His (wild-type)	Arg	Asn	Ala
k_{cat} (s^{-1})	17.5 ± 0.2	1.99 ± 0.21	2.13 ± 0.12	1.34 ± 0.84
$K_{m\ NAD^+}$ (mM)	0.087 ± 0.008	1.09 ± 0.21	1.88 ± 0.33	2.14 ± 0.38
$K_{m\ Thr}$ (mM)	3.03 ± 0.20	31.2 ± 6.1	328 ± 38	417 ± 50
$K_{i\ NAD^+}$ (mM)	0.436 ± 0.059	0.780 ± 0.165	1.340 ± 0.366	0.935 ± 0.303
$k_{cat}/K_{m\ NAD^+}$ ($M^{-1}\ s^{-1}$)	$(2.0 \pm 0.2) \times 10^5$	1825 ± 174	624 ± 79	1135 ± 149
$k_{cat}/K_{m\ Thr}$ ($M^{-1}\ s^{-1}$)	$(5.8 \pm 0.3) \times 10^3$	63.7 ± 6.2	6.50 ± 0.44	3.21 ± 0.21

Note. All initial rates were determined in duplicate at 37°C in 0.2 M Tris–HCl buffer (pH 8.4). Further details are given under Materials and Methods.

added to the mutant enzymes, they had only 59–73% of their initial activity. Mutant H90A was inactive after incubation with Mn^{2+} plus 2-mercaptoethanol while mutants H90N and H90R displayed only 5 and 29%, respectively, of their basal level of activity. Mn^{2+} alone did not greatly affect the activity of mutants H90R or H90A but reduced that of mutant H90N. 2-Mercaptoethanol, on the other hand, slightly stimulated wild-type TDH and mutant H90R but partially inhibited H90N and H90A.

Inactivation of native TDH and the Cd^{2+}-stimulated enzyme by MNBS. TDH incubated with MNBS undergoes a pseudo-first-order decay in enzymatic activity concomitant with selective methylation of His-90 (13). In the work reported here, we found that native TDH was inactivated by 2 mM MNBS, with a rate constant of 0.0093 min^{-1}, in agreement with a previous report of 0.01 min^{-1} (13). The rate constant obtained for inactivation of the Cd^{2+}-stimulated enzyme, however, turned out to be 0.043 min^{-1}.

Steady state kinetic analyses. Like wild-type TDH, all three mutants of His-90 catalyzed a sequential reaction mechanism as determined by the KinetAsyst program; Table III lists the kinetic constants derived by KinetAsyst. The k_{cat} values for all three mutants were 8–12% of the wild-type value; the K_m values for NAD^+ increased 13- to 25-fold relative to wild-type TDH, and the value of k_{cat}/K_m for NAD^+ decreased 110- to 321-fold for the mutant enzymes. The K_m values for threonine increased 10-, 108-, and 138-fold, respectively, while the k_{cat}/K_m value with respect to threonine decreased 91-, 892-, and 1807-fold, respectively, for the H90R, H90N, and H90A mutants. As calculated by the KinetAsyst program, the dissociation constant ($K_i = K_D$) for NAD^+ was generally unaffected for the mutants, with the largest change being a 3-fold increase in this value for mutant H90N.

Destabilization of the transition state in mutant H90A. As estimated from the value of $\Delta\Delta G^{\ddagger}$ between wild-type TDH and mutant H90A, the presence of His-90 in the wild-type enzyme contributes 6.6 kcal/mol of stabilization energy in the transition state of catalysis relative to its absence in mutant H90A.

Inhibition of wild-type TDH and mutant H90R by NADH. Since mutation of His-90 leads to significant increases in the K_m values for NAD^+, the binding affinity of NADH as judged by its inhibitory effect on the reaction catalyzed by wild-type TDH as well as by mutant H90R (i.e., the mutant having the largest side chain), was tested. NADH was found to be a competitive inhibitor against NAD^+ for both of these enzyme forms. Plots of $1/v_o$ vs $1/[NAD^+]$ at various levels of NADH intersected on the ordinate (data not shown) and showed K_i values for NADH of 50 ± 11 μM for wild-type TDH vs 47 ± 5 μM for mutant H90R, which values are similar to the previously reported apparent K_D value of 42 ± 7 μM for NADH with wild-type TDH (13).

Substrate specificities of the His-90 TDH mutants. The relative reactivities of the three His-90 mutants with fixed levels of various substrates and comparable data for wild-type TDH (18) are shown in Fig. 3. For wild-type TDH, L-threonine amide is about twice as reactive as L-threonine, L-threonine methyl ester, or DL-2-amino-3-hydroxypentanoate at the concentrations tested; DL-threonine hydroxamate is much less effective. In contrast, L-threonine is a poor substrate for mutants H90N and H90A, but L-threonine amide and L-threonine methyl ester are 33- to 35-fold more reactive for mutant H90A and 9- to 13-fold more reactive for mutant H90N than is threonine. Threonine hydroxamate is also a better substrate than L-threonine for mutants H90A and H90N. The best substrate for mutant H90R is L-threonine methyl ester; L-threonine is about 70% as reactive and L-threonine amide is only 40% as effective as is L-threonine. DL-2-Amino-3-hydroxypentanoate is less effectively utilized by all three mutants.

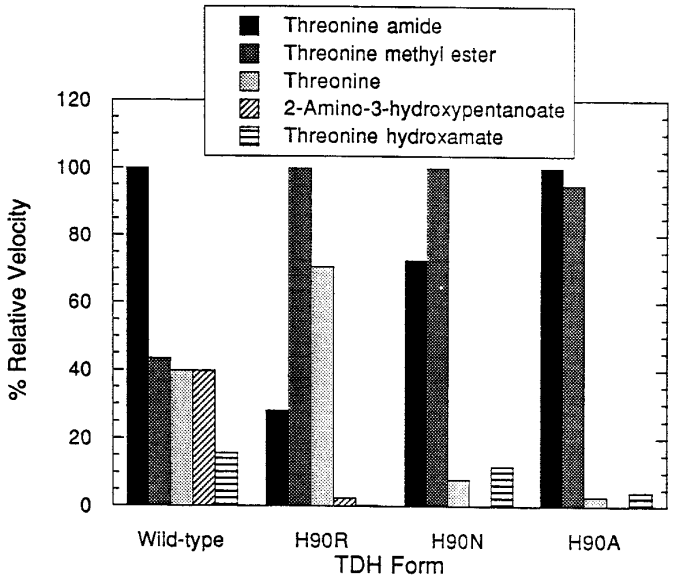

FIG. 3. Substrate specificity of wild-type TDH and the His-90 mutants. Duplicate assays (the average values are shown) were carried out at 37°C in 0.2 M Tris–HCl buffer (pH 8.4); deviation between assays was <15%. Reaction mixtures for wild-type TDH contained 125 mM substrate and 5 mM NAD^+ (18), whereas those for the His-90 mutants used 15 mM NAD^+ and the level of substrate listed under Materials and Methods. The most reactive substrate for each enzyme form was given a relative reactivity of 100%.

DISCUSSION

The site-directed mutants of *E. coli* TDH described in this report were constructed to probe the cause for the inactivation of TDH by selective methylation of His-90 (13). Some possibilities were that His-90 might be (i) a ligand to the Zn^{2+} atom of TDH, (ii) an acid/base catalyst, (iii) involved in critical hydrogen bond phenomena, or (iv) a participant in substrate recognition. In this regard, H90R, H90N, and H90A mutants of TDH were prepared, purified to homogeneity from cell-free extracts, and characterized.

These His-90 mutants showed no apparent gross conformational alterations. Each mutant enzyme was obtained in homogeneous form by the same fractionation procedures routinely used to purify wild-type TDH, and all three, like wild-type TDH (2, 13), were homotetrameric proteins as determined by their migration in nondenaturing PAGE as well as SDS–PAGE. The CD spectra of the three mutant proteins were almost identical to that of wild-type TDH. When subjected to controlled denaturation at 60°C, mutant H90R showed slightly greater, but the H90N and H90A mutants somewhat lesser, stability than wild-type TDH. Each mutant, like wild-type TDH, was also found to contain one Zn^{2+} atom/subunit, indicating that His-90 is not a ligand for the zinc atom present in the molecule. Recent X-ray absorption spectroscopic studies of *E. coli* TDH have, in fact, shown that its Zn^{2+} atom is liganded by four cysteine residues (5).

The pH-activity profiles of wild-type TDH and all three mutants are nearly identical, suggesting that His-90 has no significant role as an acid/base catalyst. If the reaction mechanism of TDH is at all comparable to that of LADH, in which His-51 (i.e., His-43 in TDH) assists in deprotonation of the alcohol substrate (15), then His-90 of TDH most likely is not a catalytic base.

In contrast to wild-type TDH whose activity is stimulated 5- to 10-fold by added Cd^{2+} or Mn^{2+} (9, 10), none of the His-90 mutants studied here is activated by these metal ions. We, therefore, investigated the possibility that His-90 might be a ligand for the Cd^{2+} ion. When wild-type TDH was first incubated with Cd^{2+} and then reacted with MNBS, no protection against inactivation was seen; on the contrary, inactivation by this methylating agent of TDH stimulated with Cd^{2+} was almost 5-fold faster than for the unstimulated enzyme. This lack of protection suggests that His-90 is not a ligand to the Cd^{2+} ion. Until the actual ligands for Mn^{2+} and Cd^{2+} in TDH are known, however, it will be difficult to interpret why these and other mutants of TDH (22) are refractory to the activating influence of metal ions. Also, as of now, no meaningful explanation can be given for the significant loss of activity that occurs when the mutant enzymes are incubated with Mn^{2+} plus 2-mercaptoethanol.

Like wild-type TDH, each mutant exhibits a sequential catalytic mechanism for the NAD^+-linked oxidation of threonine; mutant k_{cat} values, however, were reduced 10-fold. Their lowered k_{cat} values and their 10- to 138-fold increased K_m values for threonine suggest that interaction of the mutants with L-threonine was perturbed. In a sequential mechanism (see Fig. 4),

$$k_{cat} = k_5k_7k_9/(k_5k_7 + k_5k_9 + k_6k_9 + k_7k_9).$$

From this relationship, it cannot be determined why the enzymatic activity of these His-90 mutants is so

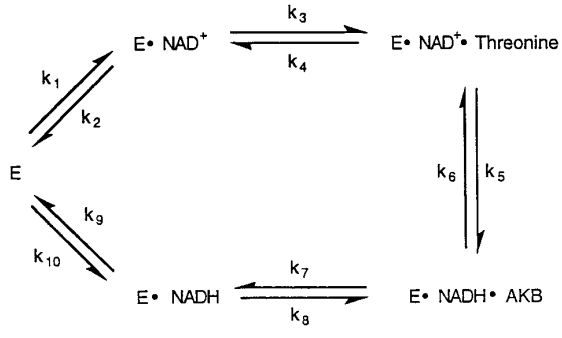

FIG. 4. Proposed sequential mechanism for the reaction catalyzed by *E. coli* TDH. E denotes the enzyme while AKB stands for 2-amino-3-ketobutyrate.

reduced. Individual steps in the complete reaction mechanism must be studied in order to see which has been affected by mutation. While a kinetic analysis of the reduction of 2-amino-3-ketobutyrate by NADH as catalyzed by the various forms of TDH would be very informative [and possibly complex due to the solution instability of 2-amino-3-ketobutyrate (23)], such studies remain to be done. Based on Fig. 4, the K_m value for threonine is not equivalent to its K_D (equal to k_4/k_3) but is composed of a complex mixture of rate constants:

$$K_{m,\ \text{L-threonine}} = k_9(k_4k_6 + k_4k_7 + k_5k_7)/$$
$$k_3(k_5k_7 + k_5k_9 + k_6k_9 + k_7k_9).$$

Hence, it cannot be concluded that the elevated K_m values for threonine are due to an increased K_D for this substrate.

On the other hand, although the K_m values for NAD^+ increased 13- to 25-fold with the mutants relative to wild-type TDH, the K_i values of all three mutants for NAD^+ were only slightly increased (a maximum of three-fold for mutant H90N). If the proposed sequential mechanism for TDH (e.g., Fig. 4) is correct, the value of k_{cat}/K_m for NAD^+ equals the second-order rate constant for binding of this coenzyme (k_1); a value for the first-order rate constant for dissociation of NAD^+ (k_2) can, therefore, be calculated since the K_D for NAD^+ (k_2/k_1) is provided by the KinetAsyst analysis. The k_2 calculated values for the various enzyme forms studied here are (s^{-1}) wild-type TDH, 87.2; mutant H90R, 1.42; mutant H90N, 0.84; and mutant H90A, 1.06. Furthermore, the K_i values of mutant H90R and wild-type TDH for the reduced coenzyme, NADH, were found to be identical in a steady-state product inhibition experiment. Hence, while the kinetics of binding of NAD^+ by the mutants may have been affected, the overall affinity of these His-90 mutants for the coenzymes appears not to be changed significantly and further shows that gross structural alterations due to the mutation have not occurred.

Although the H90R, H90N, and H90A mutants are all quite unreactive with L-threonine, mutants H90A and H90N show good catalytic activity with other substrates, indicating that the position of crucial active-site residues in these mutant proteins is not adversely affected. Although reactivity of the various TDH forms was probed at only a single, high level of each substrate, some relative information can be gleaned from these data. The altered substrate specificity of mutants H90A and H90N indicated that, although threonine and threonine hydroxamate—which have negatively charged α-carboxylate and α-hydroxamate substituents at pH 8.4, at which the kinetic measurements were taken—were poor substrates, threonine methyl ester and threonine amide (with uncharged α-carboxymethyl ester and α-carboxamide substituents) were excellent substrates for these two mutants. Mutant H90R, on the other hand, reacted best with the hydrogen bond-accepting substrates, threonine methyl ester and threonine; a hydrogen bond-donating substrate like threonine amide was less reactive than threonine in this case. While the low reactivity of mutant H90R with threonine hydroxamate is puzzling, steric factors caused by the Arg-90 side chain of this mutant may adversely affect its catalytic activity with this substrate.

The absence of a moiety in position 90 of mutant H90A that can hydrogen bond to the substrate, therefore, may contribute to its poor turnover of threonine. Based on the value of $\Delta\Delta G^{\ddagger}$ between wild-type TDH and mutant H90A, the apparent energy of activation for catalysis with mutant H90A is elevated by 6.6 kcal/mol. Since a hydrogen bond to a charged group can contribute stabilization energy on the order of 3 to 6 kcal/mol (24), destabilization of the transition state in mutant H90A due to its inability to form a hydrogen bond with threonine may be important. This is consistent with the finding that TDH mutant H90A reacted 33- to 35-fold faster with 0.75 M threonine methyl ester and threonine amide than with this concentration of threonine. Whereas threonine, threonine hydroxamate, and 2-amino-3-hydroxypentanoate have negatively charged α-carboxylate and α-hydroxamate substituents and are poor substrates for mutants H90A and H90N, this negative charge is absent in threonine amide and threonine methyl ester, both of which react well with mutants H90A and H90N. Without a hydrogen bond between such substrate analogs and His-90 (i.e., in mutant H90A), the negatively charged side chain of Glu-88 may bind well with the amino group of the substrate analog while the neutralized carboxamide or carboxyl methyl ester might possibly fill the void left by the absence of the proton-containing His-90 side chain. On the other hand, the carboxylate of threonine may be electrostatically repelled by the γ-carboxylate of Glu-88.

Other work in our laboratories has indicated that residues near His-90 in TDH also influence substrate turnover. Whereas TDH mutant E88K is catalytically inactive (22), mutant G89D is fully active (k_{cat} = 19 s^{-1}) but has an apparent K_m value for threonine of 231 mM (18). Possibly, Glu-88 interacts with the amino group of threonine while His-90 forms a hydrogen bond with the carboxylate of this substrate or Glu-88 might orient His-90 in a position optimal for binding threonine. In mutant E88K, the positively charged Lys-88 residue could repulse the amino group of threonine. While mutant G89D retains both Glu-88 and His-90, the extra negative charge from Asp-89 near the substrate binding site may decrease the affinity of this mutant for threonine.

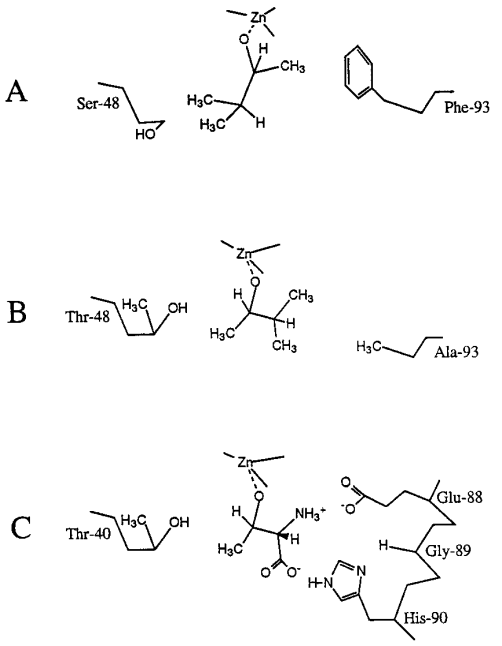

FIG. 5. Speculative drawing of the *E. coli* TDH active site (C) based on horse LADH (A) and human ADH α (B). A utilizes the crystal structure of the horse LADH·dimethyl sulfoxide·NADH complex (30) while B for human ADH α uses this structure as its model (28). In the sketch of TDH (C), L-threonine (2*S*, 3*R*) is oriented like (*R*)-(−)-3-methyl-2-butanol in B since both human ADH α and TDH use (*R*) alcohol substrates; (*S*)-(+)-3-methyl-2-butanol was previously modeled into horse LADH (28).

Relationship of His-90 of TDH with homologous dehydrogenases. His-90 of TDH is not a conserved residue among any of the homologous alcohol/polyol dehydrogenases. A histidine residue which aligns with His-90 of TDH is, however, present in XylW from *Pseudomonas putida*, but the function of this protein has not yet been determined (25). The influence of Thr-94 of LADH, which residue is analogous to His-90 of TDH, on catalysis of that enzyme is not completely known. In LADH as well as in human liver ADH β_1, however, Phe-93, which aligns with Gly-89 of TDH, controls the reactivity of primary vs secondary alcohols as well as the stereospecificity of the hydride transfer (26–28). Phe-93 and Thr-94 of human liver ADH β_1 (both of which residues are also present in horse LADH) have been changed to Ala-93 and Ile-94 to mimic the residues found in human liver ADH α (29). This mutant, β_1F93A/T94I, was shown to react selectively with secondary alcohols just like human liver ADH α. The stereochemistry of the hydride transfer in β_1F93A/T94I is reversed relative to the β_1 isozyme; the double mutant catalyzes the oxidation of (*R*) alcohols more efficiently than their (*S*) enantiomers. The stereochemical specificity of human liver ADH α is the same. TDH similarly utilizes only (3*R*) substrates such as L-threonine (2*S*, 3*R*) and D-*allo*-threonine (2*R*, 3*R*) but does not react with D-threonine (2*R*, 3*S*) or L-*allo*-threonine (2*S*, 3*S*) (2, 18).

Active site model for E. coli TDH. Figure 5 presents a speculative drawing of the active site of TDH as well as representations of two homologous enzymes. The site for LADH shown in Fig. 5A is based on the X-ray structure of this enzyme crystallized in the presence of NAD(H) and dimethyl sulfoxide (30), while the catalytic center for human liver ADH α (28) (shown in Fig. 5B) replaces Ser-48 and Phe-93 of LADH with Thr-48 and Ala-93, respectively. The sketch for the active site of *E. coli* TDH (Fig. 5C) was made based on the alignment of Phe-93 of LADH with TDH residue Gly-89 (6, 7) and includes Glu-88 and His-90. With L-threonine (2*S*, 3*R*) modeled, it can be seen that the approach of the carboxylate of the substrate to His-90 may bring Glu-88 near to its amino group (Fig. 5C). This model is compatible with the equal reactivity shown by wild-type TDH with L-threonine (2*S*, 3*R*) and D-*allo*-threonine (2*R*, 3*R*) (2); simulation of D-*allo*-threonine by inversion of the C-2 hydrogen of L-threonine would not be expected to drastically perturb any interaction between the substrate, Glu-88, and His-90. Although depiction of Zn liganded to threonine in Fig. 5C may appear contrary to the one Zn^{2+} atom of *E. coli* TDH being structural rather than catalytic (5), the model presented is consistent with the His-90 mutagenesis results detailed in this report, with data obtained for mutants E88K and G89D (18), and with unpublished findings which suggest a possible second (e.g., catalytic) Zn^{2+} site in *E. coli* TDH.

These first studies with site-directed mutants of *E. coli* TDH indicate that His-90 influences substrate reactivity. A hydrogen-bonding moiety at position 90 is not essential for catalytic activity; mutant H90A, although quite unreactive with threonine, effectively catalyzes the oxidation of threonine methyl ester and threonine amide. Studying the substrate specificity of other site-directed mutants of His-90 and Glu-88 would be helpful to test the active site model proposed here for *E. coli* TDH. If substrate-binding roles of His-90 and/or Glu-88 of TDH are established by additional data, rational alteration of the substrate specificity of TDH (i.e., to generate a general alcohol or a lactate dehydrogenase) may be possible.

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