Proton Transfer in Benzyl Alcohol Dehydrogenase during Catalysis: Alternate Proton-Relay Routes†

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ABSTRACT: His51 in horse liver alcohol dehydrogenase (ADHE) has been proposed to act as a proton donor/acceptor in the NAD^+/NADH-dependent oxidation/reduction of alcohol/aldehyde. The residue corresponding to His51 of ADHE is Val51 (Val45 in the protein sequence) in benzyl alcohol dehydrogenase (BADH) encoded by TOL plasmid pWW0. The 3-D structure of BADH modeled from the crystal structure of ADHE suggests that His47 (His41 in the protein sequence, corresponding to Arg47 in ADHE) of BADH would play the role of His51 in ADHE. To test this hypothesis, mutants of BADH, in which His47 was replaced by Gln(His47Gln) and/or Val51 was replaced by His (Val51His), were constructed. The k_{cat}/K_m value of the His47Gln mutant for benzyl alcohol was 125-fold lower than that of wild-type BADH, while the k_{cat}/K_m value of the His47Gln/Val51His double mutant was 12-fold higher than that of the His47Gln mutant. The k_{cat}/K_m value of the His47Gln mutant increased with increasing concentration of exogenous amines. These results suggest that His47 in wild-type BADH, exogenous amines in the His47Gln mutant, and His51 in the double mutant act as a general base catalyst during alcohol oxidation.

TOL plasmid pWW0 from soil bacterium *Pseudomonas putida* encodes the metabolic pathway for the degradation of toluene, xylenes, and their alcohol and carboxylate derivatives (*1*). Benzyl alcohol dehydrogenase (BADH) in this pathway oxidizes benzyl alcohol to benzaldehyde with the concomitant reduction of NAD^+. The primary structure of BADH deduced from the nucleotide sequence shows that this enzyme belongs to the zinc-containing long-chain alcohol dehydrogenase (ADH) family. BADH is a dimer with identical subunits of 38.5 kDa and contains two zinc atoms per subunit (*2, 3*). BADH, while sharing 31% identical residues with horse liver alcohol dehydrogenase (ADHE), contains several amino acid substitutions near the active site. In ADHE, His51 has been proposed to act as a general base that abstracts a proton from the alcohol substrate through a proton-relay system connecting OH of alcohol, Oγ of Ser48, O2′ of nicotinamide ribose, and Nϵ2 of His51 (*4, 5*).

The substitution of amino acid residues that are important for acid- or base-catalyzed reactions generally inactivate enzyme activity; the Lys258Ala mutant of *Escherichia coli* aspartate aminotransferase, for example, is virtually inactive. However, the activity of this enzyme has been partially restored by the addition of amines (*6, 7*), the degree of restoration being proportional to the concentration of the amine added. The efficacy of amines for this restoration differed according to the amine, and a correlation between the logarithmic value for the efficacy and pK_a of the amines (the Brønsted relationship) was observed. These results suggest that exogenous amines would substitute the functional role of Lys258. This approach has been applied to Lys296-substituted rhodopsin (*8*), Lys80-substituted leucine dehydrogenases (*9*), and Lys329-substituted ribulose 1,5-bisphosphate carboxylase/oxygenase (*10*). A mutant of human β_1 ADH, His51Gln, exhibited 6 times less catalytic efficiency than that of the wild-type enzyme. The activity of the mutant enzyme was restored by exogenous glycylglycine, glycine, and phosphate buffers (*11*). A Brønsted plot of the restoration effect versus the pK_a value of these compounds was linear, suggesting that His51 acted as a general base catalyst during the alcoholic oxidation in the wild-type enzyme.

Although residue 45 of BADH corresponding to His51 of ADHE is Val, the concentration and pK_a value of solvent proton acceptors had no effect on the catalytic efficiency of the enzyme (*3*). Therefore, it could be expected that the proton-relay system may be rearranged and connected to another internal general base in BADH. In the present study, a 3-D model of the active site of BADH was constructed on the basis of the crystal structure of ADHE (*12*). This model suggested that His47 of ADHE was located at such a position as to make a hydrogen bond with NAD ribose. We then analyzed the role of His41 of BADH (corresponding to His47 of ADHE, the residue numbering of BADH according to ADHE is used below) by site-directed mutagenesis.

MATERIALS AND METHODS

Chemicals. NAD^+ and NADH were purchased from Sigma (St. Louis, MO), $Vent_R$ DNA polymerase from New England Biolabs (Berverly, MA), synthetic oligonucleotides

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from JBioS (Niza, Japan), and the other chemicals from either Aldrich (Milwaukee, WI) or Wako Pure Chemical Industries (Tokyo, Japan).

Polymerase Chain Reaction. PCR amplification was performed in a DNA Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT) by using $Vent_R$ DNA polymerase according to the manufacturer's instructions. The PCR products were separated on 1.0% (w/v) SeaKem GTG agarose (FMC Bioproducts, Rockland, ME) or 2.5% (w/v) NuSieve GTG agarose (FMC Bioproducts) gel, and visualized by the ethidium bromide staining. The DNA bands were purified by using a QIAEX gel extraction kit (QIAGEN, Chatsworth, CA).

DNA Sequencing and Analysis. DNA sequencing was carried out by using a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, and the products were separated and analyzed by a 373 DNA sequencer (Applied Biosystems). The computer-assisted sequence analysis was done with the PC/GENE software package (IntelliGenetics, Mountain View, CA).

Bacterium, Plasmids, and DNA Manipulation. E. coli JM101 [F′ *traD36 lacI*^{q} *lacZ*Δ*M15 proA*$^+$*B*$^+$*/supE thi* Δ-(*lac-proAB*)] was used as a host cell for protein expression. All the techniques for DNA manipulation have been previously described (*13*). The *xylB* gene, the structural gene for BADH, was PCR-amplified from the pGSH2873 plasmid (*14*) by using two primers: primer I (5′-CTCGAATTCAGTGAGATAAATAGGTTGTTGT-3′) and primer II (5′-CTCGGATCCAACAACACATTCTCTACGCAA-3′). At the 5′-end of primer I, one *Eco*RI site exists, the rest of the primer sequence corresponding to the sequence upstream of the ribosome-binding site of *xylB*. At the 5′-end of primer II, one *Bam*HI site exists, the rest of the primer sequence being complementary to the sequence downstream of the termination codon of *xylB*. The amplified fragment was subcloned into pUC18 after being digested by restriction endonuclases *Eco*RI and *Bam*HI. The plasmid containing wild-type *xylB*, named pXB930, was thus constructed.

Site-Directed Mutagenesis. Point mutations, His47Gln and Val51His, were introduced into the *xylB* gene by an overlap extension PCR method (*15*), using pXB930 as a template. The mutant clones thus constructed were sequenced to confirm the presence of the correct mutations and the absence of any unexpected mutations.

Model Building. The amino acid sequences of BADH (*3*) were aligned to ADHE by using the molecular modeling program MOL-GRAPH (Daikin Industries, Tokyo, Japan), and seventeen residues around the substrate-binding cleft in the ternary ADHE-NAD^+−DMSO complex (*12*) were replaced by the corresponding amino acid residues of BADH, keeping the coordinates of the main-chain atoms unchanged. The side-chain conformations were adjusted to avoid any unfavorable contacts. Thus, the following substitutions were introduced into the 3-D structure of ADHE-NAD^+−DMSO: Ser48Thr, Leu57Tyr, Leu141Phe, Val294Ala, Ile318Val, Leu116Pro, Phe110Ser, Thr94Tyr, Asp115Gly, Asn114Ser, Pro119Gly, Ser117Asn, Ser48Thr, Val58Pro, Ala65Phe, His51Val, Thr56His, and Arg47His. After the replacement of these residues, the model was energy minimized by using the AMBER program (*16, 17*), and substrates were fitted into the active site at the most stable position with the program package, GREEN (*18*).

Enzyme Assays. Enzymatic activity was determined by following the NADH formation at 340 nm ($\epsilon = 6.22$ mM^{-1} cm^{-1}) with a Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA). The Michaelis−Menten kinetic parameters of the enzymes were measured at 25 °C in 100 mM glycine/NaOH (pH 9.4), and various concentrations of benzyl alcohol and NAD^+ were added to the reaction mixture. The dependence of the activity of BADH on the concentration (1−20 mM) and pK_a values of ammonia, methylamine, ethylamine, propylamine, butylamine, imidazole, aminoacetonitrile, aminopropyonitril, ethanolamine, trimethylamine, 2-amino-2-(hydoxymethyl)-1,3-propandiol, and 2-amino-2-methyl-1-propanol were measured under the same conditions, except that the concentration of NAD^+ was fixed at 2.5 mM. Amine stock solutions were titrated before use to pH 9.4 with sodium hydroxide or hydrochloric acid.

Protein Purification. The *xylB* product was purified from cells of *E. coli* JM101 containing a *xylB* recombinant plasmid which had been grown overnight in 500 mL of L-broth supplemented with ampicillin (50 μg/mL) and 1 mM isopropyl-β-D-thiogalactoside. The culture was centrifuged at 7700*g* for 20 min at 4 °C, the resulting pellet being resuspended in 200 mL of a 20 mM potassium phosphate buffer (pH 7.4) and the solution centrifuged again at 7700*g* for 20 min at 4 °C. The resulting pellet was resuspended in 50 mL of the 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM dithiothreitol (buffer A), and the cells were lysed by their passage through a precooled French press (Ohtake Works, Tokyo, Japan) with a pressure difference of 76 MPa. The resulting extract was centrifuged at 3000*g* for 20 min at 4 °C, the supernatant then being centrifuged at 165000*g* for 60 min at 4 °C. The resulting supernatant was passed through a Nalgen filter (0.45 μm pore size), and the filtrate was loaded into an anion-exchange HPLC column (TSKgel DEAE−5PW, 55 mm i.d. × 20 cm; Tosoh, Tokyo, Japan) that had been preequilibrated with buffer A. Proteins were eluted, after a 150-mL wash, by a gradient of 0 to 0.8 M Na_2SO_4 in buffer A at a flow rate of 20 mL/min, and each 10-mL fraction was collected. Those fractions containing BADH activity were pooled and brought up to 1 M in $(NH_4)_2SO_4$ at 4 °C, the resulting suspension being centrifuged at 7700*g* for 20 min. BADH activity was recovered in the supernatant. The resulting supernatant was passed through the Nalgen filter and loaded into a hydrophobic interaction chromatography column (TSKgel Phenyl-5PW, 21.5 mm i.d. × 15 cm; Tosoh) that had been preequilibrated with buffer A containing 1 M $(NH_4)_2SO_4$. After washing with 75 mL of the same buffer, the column was eluted at a flow rate of 5 mL/min by using a linear gradient of $(NH_4)_2SO_4$ from 1.0 to 0.1 M over a period of 20 min and then a linear gradient of 2-propanol from zero to 10% (v/v) over a period of 60 min. Each 5-mL fraction was collected, and those fractions containing BADH activity were pooled.

Amino-Terminal Sequencing. The amino-terminal sequences of the purified enzymes were determined by Edman degradation with an automated protein sequencer (model 477, Applied Biosystems).

Analytical Methods. SDS−PAGE was performed with premade polyacrylamide slab gel plates (MultiGel 4/20; Daiichi Pure Chemicals, Tokyo, Japan). Proteins were

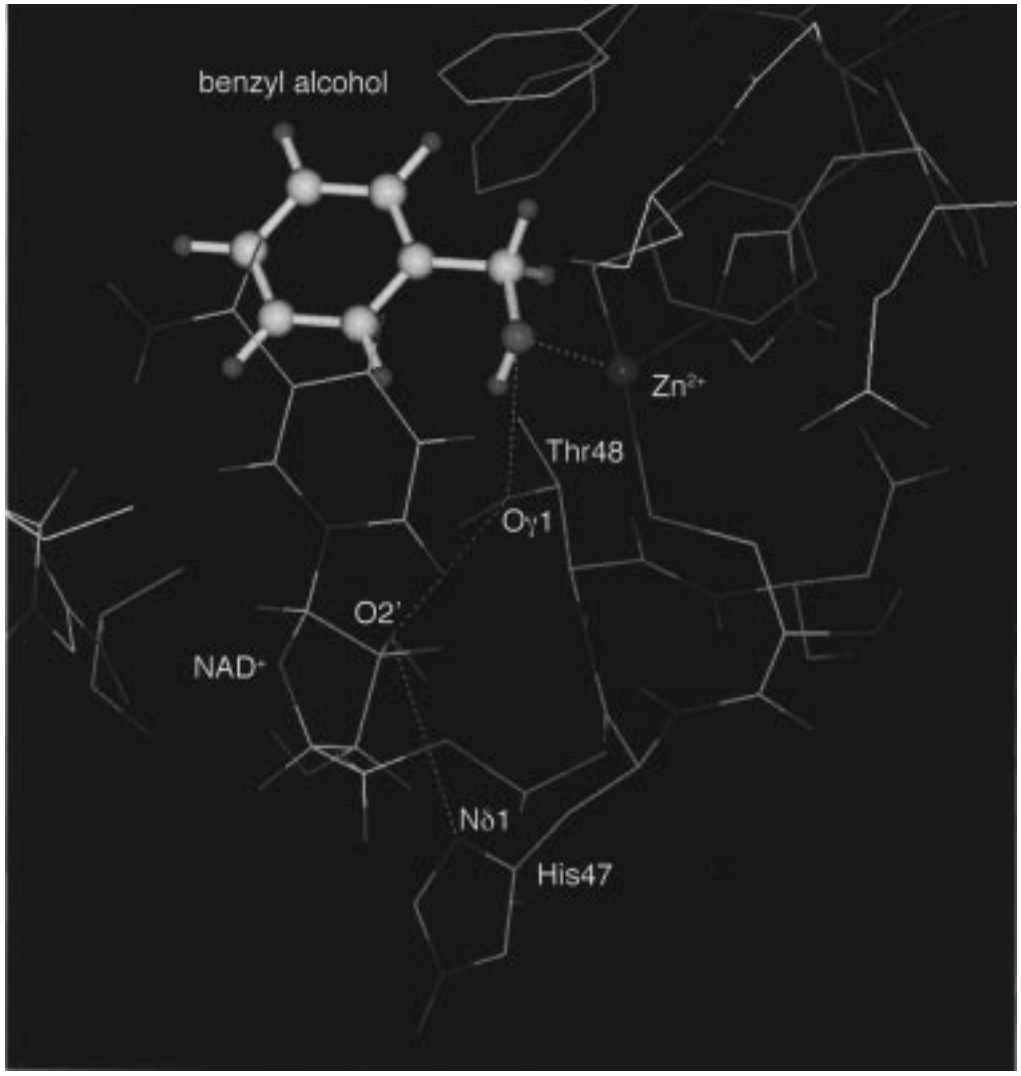

FIGURE 1: Hydrogen bond system in the active-site model of benzyl alcohol dehydrogenase. The system comprises benzyl alcohol (shown as a ball-and-stick model), nicotinamide ribose, Thr48, and His47. An ionic or hydrogen bond (distance < 0.35 nm) is shown between Zn → O of benzyl alcohol, O of benzyl alcohol → Oγ1 of Thr48, Oγ1 of Thr48 → O2′of NAD^+, and O2′of NAD^+ → Nγ1 of His47. Val51 is located above of the plane, and is not shown in this figure.

stained for 1 h with 0.1% (w/v) Coomassie brilliant blue R250 in an aqueous solution containing 50% (v/v) methanol and 10% (v/v) acetic acid, and destained in a solution containing 10% (v/v) methanol and 10% (w/v) acetic acid. The protein concentration was determined according to the method of Bradford (*19*), using an assay kit from Bio-Rad Japan (Tokyo, Japan) and bovine serum albumin as a standard.

RESULTS

Model Construction. The identity in amino acid sequence between ADHE and BADH was 30%. Although the zinc-liganding residues (Cys and His) were conserved in both enzymes, six of the eight residues around the active site at positions 48, 141, 57, 116, 294, and 318 (numbered according to ADHE) were substituted between these enzymes. The 3-D structural model of the substrate-binding pocket of BADH was built from the crystal structure of ADHE by replacing 17 residues of ADHE around the catalytic center with the corresponding residues of BADH. The model (Figure 1) suggests that His47 was located at the surface of the protein. The distance between the Nδ1 atom of His 47 and the O2′ atom of the NAD^+ ribose moiety was calculated to be 0.35 nm, this being close enough to form a hydrogen bond between them. Therefore, it was possible for His47 to be served as a terminal proton donor/acceptor of the proton-relay system in BADH.

Construction of Mutant BADHs. To test the hypothesis of His47 acting as a proton donor/acceptor, the mutant genes synthesizing the His47Gln and Val51His enzymes were constructed, and the wild-type and mutant BADHs expressed in *E. coli* were purified. Approximately 2−5 mg of the purified BADHs were obtained per liter of the bacterial cultures and they displayed a single band by SDS−PAGE. The amino-terminal sequences of these proteins were determined to be MEIKAAIVR, this sequence fitting the amino-terminal sequence predicted from the *xylB* nucleotide sequence (*3*).

Steady-State Kinetic Characterization. To characterize the functional properties of the recombinant enzymes, their steady-state kinetics for the oxidation of benzyl alcohol were examined (Table 1), and the k_{cat}/K_m values, which represent the overall catalytic rate constant, were compared. The k_{cat}/K_m value of the His47Gln mutant was 41 s^{-1} mM^{-1}, which

Table 1: Steady-State Kinetic Constants of the Wild-type and Mutant BADHs

constant[a]	wild-type	His47Gln	His47Gln/Val51His
	Forward Reaction		
K_{cat} (s^{-1})	164	33	18
K_m (benzyl alcohol) (mM)	0.032	0.822	0.037
K_{cat}/K_m (benzyl alcohol) (s^{-1} mM^{-1})	5130	41	486
K_m (NAD^+) (mM)	0.144	0.139	0.020
	Reverse Reaction		
K_{cat} (s^{-1})	1470	25	62
K_m (benzaldehyde) (mM)	0.122	0.81	0.08
K_{cat}/K_m (benzaldehyde) (s^{-1} mM^{-1})	12 000	30	780
K_m (NADH) (mM)	1.1	0.096	ND[b]
K_i (NADH) (mM)	0.62	0.18	ND

[a] The kinetic constants for the forward (NAD^+-dependent oxidation of benzyl alcohol) and reverse (NADH-dependent reduction of benzaldehyde) reactions were determined in 100 mM Glycine/NaOH (pH 9.4). [b] Not determined.

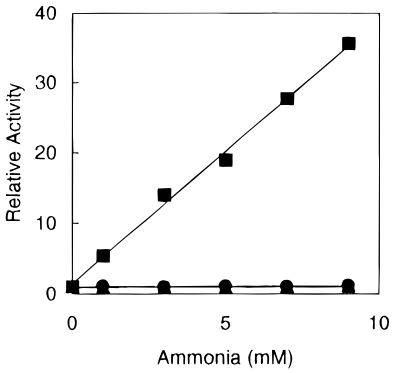

FIGURE 2: Effect of ammonia on the speed of oxidation of benzyl alcohol by the wild-type, His47Gln and His47Gln/Val51His enzymes of BADH. The k_{cat}/K_m values were determined at each ammonia concentration in a 0.1 M Glycine/NaOH buffer (pH 9.4) with 2.5 mM NAD^+. The activities of the wild-type (●), His47Gln (■), and His47Gln/Val51His (▲) enzymes in the absence of ammonia were taken as 1.

is about 125-times less than that of the wild-type enzyme (5130 s^{-1} mM^{-1}). However, the introduction of the Val51His mutation into the His47 enzyme restored the catalytic efficiency by up to 12-fold (486 s^{-1} mM^{-1}).

The kinetic constants of BADHs for the reduction of benzaldehyde (the reverse reaction) were also determined at pH 9.4 (Table 1). In the reverse reaction too, the k_{cat}/K_m value of the His47Gln mutant was 400-fold smaller than that of the wild-type enzyme, and the k_{cat}/K_m value of the His47Gln/Val51His mutant was 26-fold higher than that of the His47Gln mutant. The K_m values of the wild-type and His47Gln enzymes for NAD^+ in the forward reaction were almost identical; however, those for NADH in the reverse reaction were quite different. This difference was partly due to the difference in the affinity of these enzymes to NADH because the K_i values for NADH of the wild-type and His47Gln enzymes were 620 and 180 μM, respectively.

Effect of Exogenous Amines on the BADH Activity. Figure 2 illustrates the dependence of the k_{cat}/K_m value on the ammonia concentration of the wild-type, His47Gln, and His47Gln/Val51His enzymes. The k_{cat}/K_m value of the His47Gln mutant increased linearly with increasing concentration of ammonia up to 20 mM, while those of the wild-type and His47Gln/Val51His enzymes remained constant. The effect of ammonia on the k_{cat}/K_m value of His47Gln was pH dependent. The ratio of the slopes at pH 8.63 and pH 9.33 in Figure 3 is 2.5 ± 0.2, which is equivalent to the ratio of the concentration of the free-base form of NH_3 at each pH value. We thus propose that the observed k_{cat}/K_m value, $(k_{cat}/K_m)_{obs}$, at an amine concentration, [amine], can be expressed by the equation:

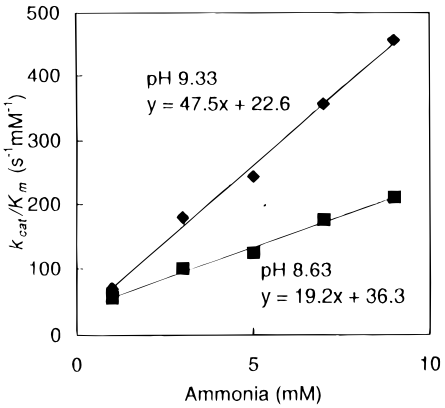

FIGURE 3: pH dependence in the activation of the His47Gln mutant of BADH by ammonia. The k_{cat}/K_m values were determined as described in Figure 2. The ratio of the slopes at pH 9.33 and 8.63 was 2.5 ± 0.2. The ratio of the concentrations of the free base at these pH values was calculated to be 2.7 when using a pK_a value of 9.2 for ammonia.

$$(k_{cat}/K_m)_{obs} = (k_{cat}/K_m)_o\left(\frac{1 + k_B[\text{amine}]_{total}}{1 + \frac{[H^+]}{K_a}}\right)$$

where $(k_{cat}/K_m)_o$ is the k_{cat}/K_m value in the absence of an amine, and k_B is a constant indicating the increase in k_{cat}/K_m value per molarity of free base. The effect of ammonia observed in Figures 2 and 3 was not a consequence of any change in ionic strength, since we obtained the same activation value for ammonia in a buffer either containing or not containing 100 mM NaCl.

Brønsted Analysis of the Activation of the His47Gln Enzyme by Amines. The k_B values of the His47Gln enzyme for eight primary amines and imidazole were determined (Table 2). Figure 4 shows a Brønsted plot, where log k_B values of the amines were plotted against their pK_a values. Nine amines exhibited a linear Brønsted relationship with a slope of 0.14 ± 0.03. A deviation from the Brønsted relationship by about 0.9 in the log k_B unit was observed with tertiary amines and those amines having bulky side chains (e.g., 2-amino-2-(hydoxymethyl)-1,3-propandiol and 2-amino-2-methyl-1-propanol). The lower efficiency of these amines in the activation of the His47Gln enzyme may have resulted from steric hindrance to their interaction with O2′ of nicotinamide ribose.

DISCUSSION

His51 (the numbering according to ADHE) is thought to act as a proton acceptor in Zn-dependent ADH, including human β_1 ADH (*5*). This proposal has been supported by the observation that a mutant enzyme of human β_1 ADH, in which His51 was replaced by Gln, had a 6-fold decreased

Table 2: Activation-Rate Constants, k_B, for the Amine-Assisted Catalysis of Benzyl Alcohol Oxidation by the His47Gln Mutant of BADH

amine	pK_a[a]	log k_B[b]
methyl-	10.6	3.46
ethyl-	10.6	3.28
propyl-	10.5	3.35
butyl-	10.6	3.40
ethanol-	9.5	3.00
ammonia	9.2	3.56
2-cyanoethyl-	7.7	2.87
imidazole	6.99	2.95
cyanomethyl-	5.3	2.62
trimethyl-	9.8	2.54
2-amino-2-methyl-1-propanool	9.7	2.61
2-amino-2-(hydroxymethyl)-1,3-propandiol	8.1	2.17

[a] Values from Sober (*30*). [b] The k_B values are expressed as inverse molarity of the free base form of amine.

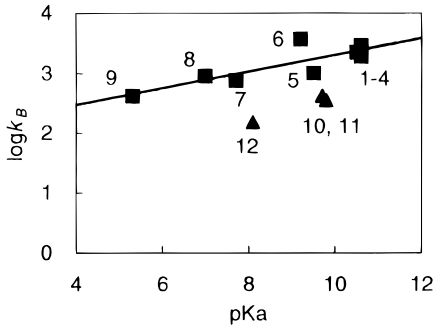

FIGURE 4: Brønsted plot for the increase in k_{cat}/K_m (s^{-1} mM^{-1}) per molarity of exogenous amines. Each data point represents the k_B value of the following amines: 1, methylamine; 2, ethyamine; 3, propylamine; 4, butylamine; 5, ethanolamine; 6, ammonia; 7, 2-cyanomethylamine; 8, imidazol; 9, cyanoethylamine; 10, trimethylamine; 11, 2-amino-2-methyl-1-propanol; 12, 2-amino-2-(hydoxymethyl)-1,3-propandiol. The line whose slope is 0.14 ± 0.03 represents a fit to all the data points except 10, 11, and 12.

k_{cat}/K_m value compared to that of the wild-type enzyme for ethanol oxidation at pH 7.0 (*11*). This decrease could be restored by the addition of exogenous glycylglycine, glycine, or phosphate anions. The explanation of this observation is that these exogenous compounds replaced the function of His51. The residue corresponding to His51 of ADHE is Val in BADH. A computer model of the active center of BADH predicted His47 to be a proton donor/acceptor in the catalytic reaction. This hypothesis is experimentally supported by the observations that the k_{cat}/K_m value for benzyl alcohol of the His47Gln mutant of BADH was about 125-fold lower than that of the wild-type, but that the defect was partially restored by the addition of such amines as ammonia. The activation of the His47Gln enzyme by ammonia was proportional to the concentration of its free-base form, indicating that the rate-limiting step of catalysis in the mutant enzyme was the release of a proton generated by the oxidation of alcohol into a solvent. No such activation by ammonia and other amines was observed in the wild-type enzyme. Thus, His47 in wild-type BADH may have acted as the terminal proton acceptor/donor in the catalysis as schematically shown in Figure 5. The His47Gln/Val51His double mutant contained a His residue introduced at the same position to His51 of ADHE. The k_{cat}/K_m value of the His47Gln/Val51His mutant was 12-times higher than that of the His47Gln enzyme, and this value was not influenced by exogenous amines. We

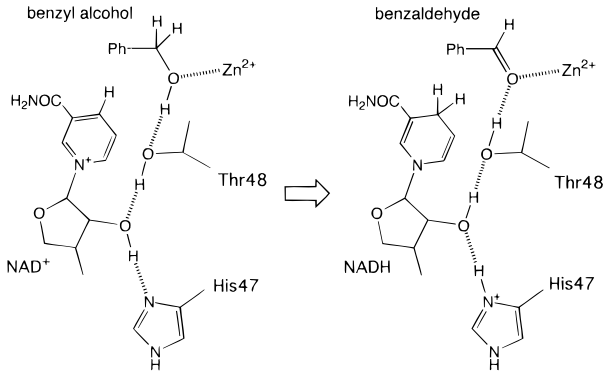

FIGURE 5: Proposed scheme for the hydrogen-bonding network in the BADH active site.

interpret this observation as indicating that His51 in the mutant acted as the terminal proton acceptor/donor in the catalysis, the efficiency of proton transfer via His51 being higher than that mediated by exogenous amines.

The activation of the His47Gln enzyme was saturated at 30 mM of free base of ammonia (data not shown), indicating either that a rate constant determining the speed of proton transfer was no longer rate limiting at that concentration of the base or that the formation of the base−enzyme complex was saturated at that concentration of the base. The activity of the His47Gln enzyme in the presence of the saturated concentrations of ammonia was lower than that of the wild-type enzyme. This observation suggested either that the ammonia-aided proton transfer in the mutant enzyme was not as efficient as the native proton-relay system, or that the His47Gln mutation caused addition lesion to the proton-relay system. Among 12 bases tested, nine formed a linear Brønsted relationship, whereas Tris and its derivatives deviated by about 0.9 in the log k_B unit (Figure 4). Such deviation from the Brønsted relationship most probably resulted from steric hindrance. In contrast to the results with aspartate aminotransferase (*6*, *7*), leucine dehydrogenase (*9*) and ribulose 1,5-bisphosphate carboxylase/oxygenase (*10*), steric factors of primary amines may not have influenced the chemical rescue of the His47Gln mutant.

Glycine and other compounds that have been shown to be effective in activating the His51Gln mutant of human β_1 ADH did not restore the activity of the His47Gln derivative of BADH (data not shown). The difference in the effectiveness of specific bases between human β_1 ADH and BADH implies a different electrostatic environment around the NAD^+-binding sites in these enzymes. Amines accept protons when they are neutral, while glycine accepts protons when it is negatively charged. It is likely that negatively charged proton acceptors such as glycine can access NAD^+ in human β_1 ADH, while only neutral forms of proton acceptors can access NAD^+ in the His47Gln mutant of BADH.

Ser48 in ADHE is also thought to be involved in the proton-relay system (*5*, *20*, *21*). Thr has been found at the corresponding position in some Zn-dependent ADHs. Computer modeling has suggested that the hydroxyl group of Thr48 in human β_1 ADH can form a similar hydrogen-bonding system to that of Ser48 (*5*). It is likely that a residue equivalent to Ser48 is always involved in the proton-relay of all Zn-dependent ADHs (*22*). The position of His51 in ADHE is not necessarily conserved among Zn-dependent

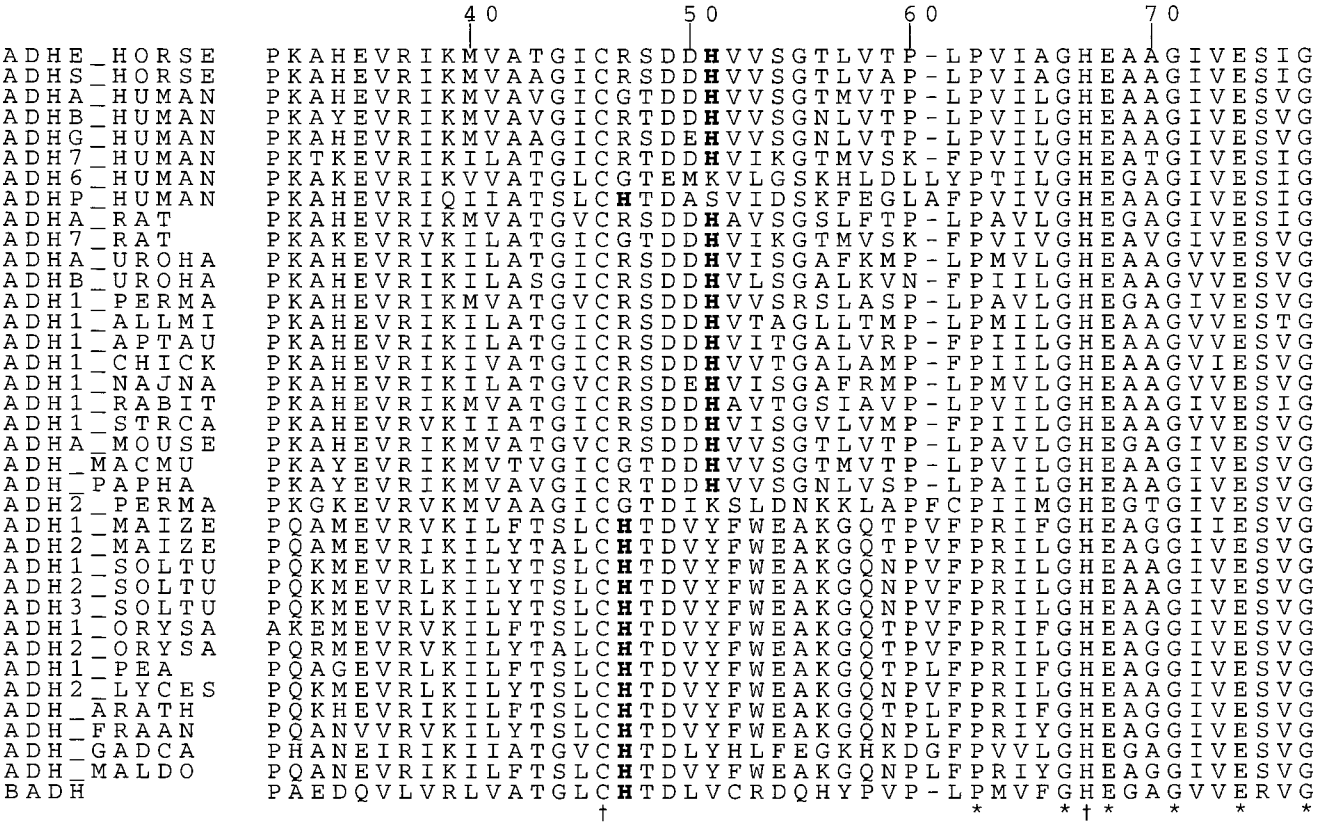

FIGURE 6: Comparison of the amino acid sequences of Zn-dependent alcohol dehydrogenases around residue 51. Numbering is according to that of ADHE_HORCE (=ADHE). Data are from the SWISS−PROT data bank. Residues marked with † bind the catalytic Zn ion. Residue 51 of ADHP_HUMAN has been reported to be either Ser or Thr (*24*).

ADHs. Substitution to Tyr, Lys, or Ser for His51 occurs frequently (*23*; Figure 6). Although it is possible that Tyr, Lys, and Ser act as terminal proton acceptors/donors, an alternative possibility is that His47 in these enzymes plays such a role. This possibility has already been discussed by Davis et al. (*24*). The class II human $\pi\pi$ ADH isozyme has Thr51, and substitution of Ser or His for Thr did not influence the enzyme activity. They pointed out that His47 is highly conserved among ADHs that do not have His51, and suggested that His47 can act as general base catalyst in these enzymes. The three-dimensional structure of β_2 ADH possessing His47 and His51 showed that His51 is involved in the proton-relay system while His47 forms a stable hydrogen bond with the adenosine phosphate of NAD^+ (*25*). The structural analysis of cod liver ADH possessing His47 and Tyr51 showed that Tyr51 is not involved in the proton transfer, and a water molecule bound to O2′ of nicotinamide ribose is close to His47 in one subunit of the enzyme. From this structural configuration, the proton-relay system from His47 to O2′ of nicotinamide ribose via a water molecule has been proposed (*26*). Similarly, His47 in the human class III $\chi\chi$ ADH forms a hydrogen bond to O2′ of nicotinamide ribose (*27*). From these observations together with our present results, it is likely that His47 in ADHs that do not have His51 is involved in the proton-relay system.

The positively charged side chain at Arg47 in ADHE interacts with the negatively charged pyrophosphate moiety of the NAD(H) molecule (*12*). With human β_1 ADH, in which the amino acid at position 47 was changed by site-directed mutagenesis, the more basic the amino acid residue at that position, the higher was the affinity for NAD^+ (*28*). Position 47 in many enzymes involves Arg, but substitution to His is frequently observed. From the analysis of an His47Arg mutant of *Saccharomyces cerevisiae* isoenzyme I, Gould and Plapp (*29*) suggested that residue 47 is dispensable for the cofactor binding, but this residue is involved in a conformational change after the coenzyme binding. In the human β_1 ADH, the substitution of Arg47 by Gly did not change the affinity to the coenzyme. The structural analysis of the variant, however, did not rule out the interaction between Arg47 and the coenzyme, but the result of the above-mentioned experiment was interpreted as indicating that the Arg47Gly substitution provokes a local structural alteration which compensates for the loss of coenzyme interaction (*25*). In BADH, the electrostatic interaction between the imidazole ring of His47 and the pyrophosphate moiety of NADH may not be significant at pH 9.4 because the affinity for NADH of the His47Gln mutant (K_i was 180 μM at pH 9.4) was higher than that of the wild-type enzyme (K_i was 620 μM at pH 9.4). However, it is clear from the alteration of K_i that residue 47 of BADH is somehow involved in the cofactor binding. The role of His47 of BADH in the cofactor binding should be carefully examined in future studies.

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