

## Cloning and Sequencing of the Gene Coding for Alcohol Dehydrogenase of *Bacillus stearothermophilus* and Rational Shift of the Optimum pH

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Using *Bacillus subtilis* as a host and pTB524 as a vector plasmid, we cloned the thermostable alcohol dehydrogenase (ADH-T) gene (*adhT*) from *Bacillus stearothermophilus* NCA1503 and determined its nucleotide sequence. The deduced amino acid sequence (337 amino acids) was compared with the sequences of ADHs from four different origins. The amino acid residues responsible for the catalytic activity of horse liver ADH had been clarified on the basis of three-dimensional structure. Since those catalytic amino acid residues were fairly conserved in ADH-T and other ADHs, ADH-T was inferred to have basically the same proton release system as horse liver ADH. The putative proton release system of ADH-T was elucidated by introducing point mutations at the catalytic amino acid residues, Cys-38 (cysteine at position 38), Thr-40, and His-43, with site-directed mutagenesis. The mutant enzyme Thr-40-Ser (Thr-40 was replaced by serine) showed a little lower level of activity than wild-type ADH-T did. The result indicates that the OH group of serine instead of threonine can also be used for the catalytic activity. To change the  $pK_a$  value of the putative system, His-43 was replaced by the more basic amino acid arginine. As a result, the optimum pH of the mutant enzyme His-43-Arg was shifted from 7.8 (wild-type enzyme) to 9.0. His-43-Arg exhibited a higher level of activity than wild-type enzyme at the optimum pH.

Various thermostable alcohol dehydrogenases (ADH-Ts) have been analyzed for the industrial production of alcohol (2, 26, 38), including chiral alcohol (20). *Bacillus stearothermophilus* NCA1503 was found to produce an ADH-T amounting to 1 to 2% of soluble cell protein. This strain produced ethanol from sucrose or glucose as a carbon source under anaerobic conditions at high temperatures (2, 27). Two types of ADH have been isolated from *B. stearothermophilus* NCA1503 and DSM2334 (33). ADH-T from NCA1503 showed enzymatic, structural, and immunological properties different from those of the ADH from DSM2334. The ADH from DSM2334 is active with primary alcohols, including methanol, and the rate-limiting step is NADH release as seen with horse liver ADH (3, 33). In contrast, substrate inhibition is not observed for ADH-T with any alcohols, and the enzyme-NADH dissociation is not considered to be a rate-limiting step (33). The gene for ADH from DSM2334 has been cloned in *Escherichia coli* (8). In this work, we cloned the ADH-T gene (*adhT*) from *B. stearothermophilus* NCA1503 in *Bacillus subtilis*.

The ADH reaction mechanism was originally studied with horse liver ADH by X-ray crystallographic analysis and kinetic studies (3, 9, 36). Catalysis by horse liver ADH occurs by a proton release system involving a zinc atom, a water molecule, and serine and histidine residues. By comparing amino acid sequences of ADH-T and other ADHs, the catalytic system of ADH-T was inferred.

We report here the molecular cloning and nucleotide sequencing of the ADH-T gene, *adhT*, from *B. stearothermophilus* NCA1503, a comparison of the deduced amino acid sequence with the sequences of other ADHs, prediction of the catalytic system of ADH-T based on the crystallo-

graphically determined model of the horse liver ADH, confirmation of the putative catalytic system by replacing the catalytic amino acids of ADH-T by using site-directed mutagenesis, and construction of a modified enzyme that exhibits a pH profile different from that of the wild-type ADH-T.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** *B. stearothermophilus* NCA1503 (34) was used as a DNA donor. *B. subtilis* MI113 (*arg-15 trpC2 hsrM hsmM*) (34) and MI112 (*leuA8 thr-5 arg-15 recE4 hsrM hsmM*) (35) were used as host cells in gene cloning. Since *B. subtilis* MI112 is deficient in DNA recombination, it was used as the host cell to stably carry the recombinant plasmid. A low-copy-number plasmid, pTB524 (coding for tetracycline resistance [Tc<sup>r</sup>]) (24), which has a *Bam*HI site suitable for gene cloning, was used to construct the gene bank of *B. stearothermophilus* NCA1503. pTB522 (Tc<sup>r</sup>) (14), which has a *Hind*III site for cloning, and pTB524 were used for subcloning of the gene. *E. coli* TG1 [*supE*  $\Delta$ (*lac-proAB*) *hsd* $\Delta$ 5 F' *traD36 proAB*<sup>+</sup> *lacI*<sup>q</sup> *lacZ* $\Delta$ M15] (31) and M13 mp18 and M13 mp19 (31) were used as a host cell and phages to subclone the gene for nucleotide sequencing.

**Media.** *B. stearothermophilus* NCA1503 was grown at 55°C in modified L broth containing tryptone (20 g/liter), yeast extract (10 g/liter), and NaCl (5 g/liter), and the pH was adjusted to 7.3 with 2 N NaOH. *B. subtilis* MI113 and MI112 were grown in L broth (34) at 37°C. Solid medium contained 20 g of agar per liter for growth at 55°C and 15 g of agar per liter for growth at 37°C. Transformants of *B. subtilis* with pTB524, pTB522, or their derivatives carrying the tetracycline resistance gene were grown in L broth containing tetracycline (25  $\mu$ g/ml).

**Detection of ADH-producing colonies on plates.** ADH-

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producing colonies were selected on modified aldehyde indicator plates as described by Conway et al. (5), with slight modification. The plates were composed of antibiotic medium 3 (17.5 g/liter) (Difco Laboratories, Detroit, Mich.) acting as a buffer (pH 7.0), ethanol (20 ml/liter), pararosaniline (50 mg/liter) (Sigma Chemical Co., St. Louis, Mo.), and sodium hydrogen sulfite (250 mg/liter). Ethanol diffuses into cells and can be converted by ADH to acetaldehyde, which reacts with the reagents to form Schiff base intense red.

**Preparation of plasmids and chromosomal DNA.** Either the rapid alkaline extraction method or CsCl-ethidium bromide equilibrium density gradient centrifugation was used to prepare plasmid DNA, whereas chromosomal DNA was prepared as described elsewhere (13, 19).

**Transformation of *B. subtilis*.** For transformation of *B. subtilis*, competent cells were prepared as described previously (19). Tc<sup>r</sup> transformants were transferred on the modified aldehyde indicator plates and incubated at 37°C for 5 h.

**Nucleotide sequencing.** DNA was sequenced by the dideoxy method of Sanger et al. (32) with the Sequenase sequencing kit (United States Biochemical Corp., Cleveland, Ohio). After digestion with restriction enzymes, DNA fragments were subcloned into M13 mp18 or M13 mp19. *E. coli* TG1 was used as a host cell.

**Site-directed mutagenesis.** Point mutations were introduced into a gene with an oligonucleotide-directed in vitro mutagenesis system (Amersham, Buckinghamshire, United Kingdom) according to the manufacturer's instructions.

**Active staining of ADH.** Active staining of ADH was performed according to the method described by Dowds et al. (8). Crude enzymes were run on a 6% polyacrylamide gel with solutions and reagents from which sodium dodecyl sulfate (SDS) was omitted. The gel was stained for ADH activity by an alcohol-dependent nitroblue tetrazolium procedure. The gel was soaked in 500 mM Tris-HCl (pH 8.8) at 4°C for 15 min and then incubated at 37°C for 30 min in a staining solution containing 150 mM Tris-HCl (pH 8.8), NAD (0.132 mg/ml), nitroblue tetrazolium (0.163 mg/ml), phenazine methosulfate (0.03 mg/ml), and ethanol (10 ml/liter). These reagents were purchased from Sigma Chemical Co.

**Enzyme purification.** ADH-T and its derivatives were purified from the transformants. Cells were grown to the stationary phase, harvested by centrifugation (10,000 × g, 10 min) at 4°C, and washed in 20 mM potassium phosphate buffer (pH 7.8). The cell pellet was suspended in phosphate buffer containing lysozyme (1 mg/ml) and DNase I (10 U/ml) and incubated at 37°C for 30 min. After centrifugation (55,000 × g, 30 min), the supernatant was heated at 60°C for 10 min and again centrifuged (20,000 × g, 10 min) at 4°C. The crude enzyme was purified by DEAE-cellulose (DE 52; Whatman BioSystems Ltd., Maidstone, Kent, England) ion-exchange column chromatography. The enzyme was eluted with a linear gradient (0 to 1 M) of potassium chloride. Active fractions were dialyzed overnight at 4°C in 20 mM potassium phosphate buffer (pH 7.8). The final enzyme preparation was confirmed to be homogeneous by SDS-polyacrylamide gel electrophoresis (PAGE). According to the method described above, wild-type ADH-T and mutant enzymes Thr-40-Ser and His-43-Arg were purified to homogeneity (data not shown).

**Assay of ADH activity.** ADH was assayed by monitoring ethanol-dependent NAD reduction at 340 nm (21, 33, 37). ADH activity was expressed as micromoles of NADH produced per minute, with a molar absorption coefficient of

6.22 mM<sup>-1</sup> · cm<sup>-1</sup>. The standard ADH assay was performed at 55°C in a reaction mixture which contained 100 mM potassium phosphate buffer (pH 7.8), 1 mM NAD, and 100 mM ethanol. To examine the pH profile of the enzyme, 100 mM potassium phosphate buffer or 100 mM glycine-KOH buffer having various pHs was used.

**Protein assay.** The protein concentration was measured by the method of Lowry et al. (22) with bovine serum albumin as the standard.

**Computer analysis of amino acid sequence homology.** Homology of primary structure was analyzed by the method described by Needleman and Wunsch (25). An NEC PC-9801RA computer (Nippon Electric Co., Tokyo, Japan) and the software DNASIS (Hitachi Software Engineering Co., Kanagawa, Japan) were used for the analysis.

**Other procedures.** Procedures for digestion of DNA with restriction endonucleases, ligation of DNA with T4 DNA ligase, agarose gel electrophoresis, and SDS-PAGE were described elsewhere (1, 10, 12, 13). Unless otherwise specified, all chemicals used in this work came from sources described in a previous paper (30).

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under the accession number D90421.

## RESULTS

**Cloning of the ADH gene from *B. stearothermophilus* NCA1503.** Chromosomal DNA of *B. stearothermophilus* was partially digested with *Sau*3AI, and fragments of approximately 6 kb were isolated and purified by Gene-Clean (Bio 101 Inc., La Jolla, Calif.). These fragments were ligated into the *Bam*HI site of pTB524. The ligation mixture was used to transform *B. subtilis* MI113. Of 3,000 Tc<sup>r</sup> transformants of *B. subtilis*, one ADH-positive clone was found on the modified aldehyde indicator plates. The transformant carried a recombinant plasmid containing an insert of about 7 kb. A lysate of the candidate cell, subjected to electrophoresis, showed a band of ADH-active staining at the same position as that of

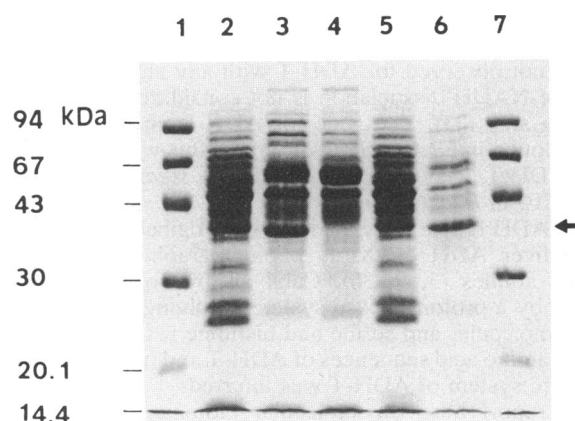


FIG. 1. SDS-PAGE analysis of cell extracts. Each lane contains 5  $\mu$ l of cell extract. Lanes: 1 and 7, molecular size markers; 2, *B. stearothermophilus* NCA1503; 3, ADH-positive transformant of *B. subtilis* MI113; 4, *B. subtilis* MI113; 5, cell extract of *B. stearothermophilus* NCA1503, heated (60°C, 10 min) and centrifuged (20,000 × g); 6, cell extract of *B. subtilis* transformant, heated (60°C, 10 min) and centrifuged (20,000 × g). The arrow indicates the position of ADH.

FIG. 3. Nucleotide sequence of the *adhT* gene and the deduced amino acid sequence of the encoded protein. The 1.7-kb *EcoRI*-*Bam*HI fragment (Fig. 2) is shown. The amino acid sequence is shown above the nucleotide sequence. A probable Shine-Dalgarno (SD) sequence is indicated by a solid line. The terminator and inverted repeat at the 5'-flanking region are shown by arrows. Asterisk indicates a stop codon.

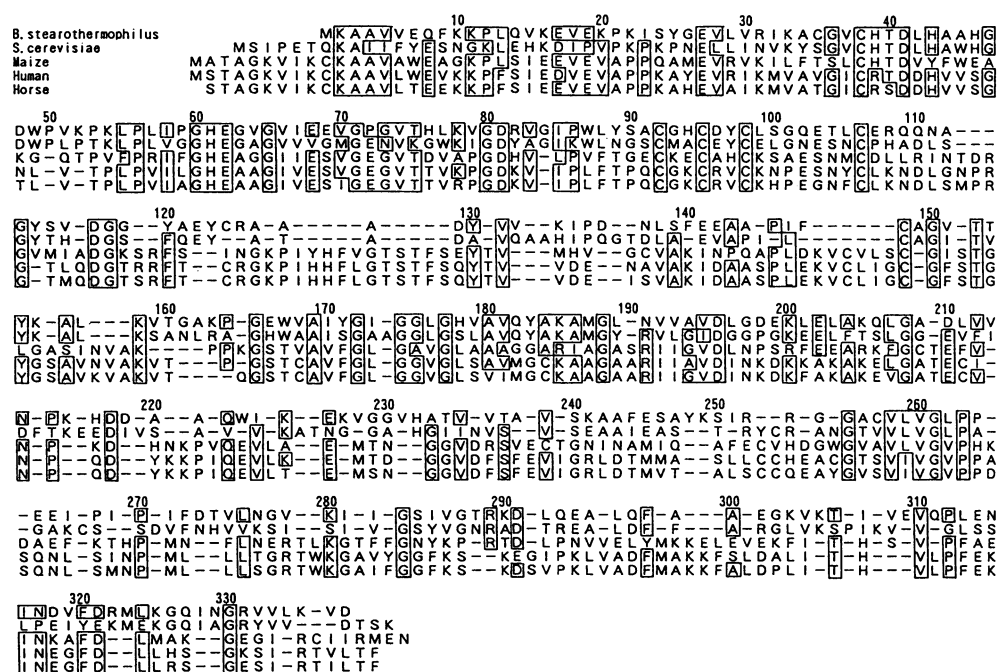


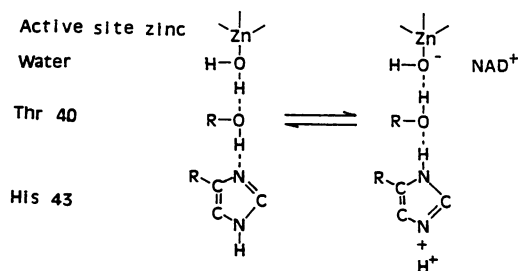
FIG. 4. Comparison of amino acid sequences of five different ADHs: *B. stearothermophilus* ADH-T (this work), *S. cerevisiae* ADHII (29), maize ADH1 (6), human ADH  $\beta$ 1 subunit (11), and horse liver ADH E subunit (17). Amino acids are numbered according to the amino acid sequence of *B. stearothermophilus*. Homologous sequences are boxed. Gaps (-) are introduced to obtain maximal matching.

**Comparison of the deduced amino acid sequence with the sequences of four different ADHs.** Comparison of the primary structures of enzymes with the same function but different origins is useful to determine the amino acids essential for activity, because the active site and substrate binding site are highly conserved (15). ADHs are widely distributed in different organisms and tissues. We compared the amino acid sequences of different ADHs. Most of them showed homology. The deduced amino acid sequence of ADH-T from *B. stearothermophilus* was homologous (45% identity) with that of *Saccharomyces cerevisiae* (29), while the degree of amino acid homology of ADH-T with enzymes from maize (6), humans (11), and horse liver (17) was about 35%. However, no homologous region between ADH-T and other bacterial ADH was found (5). The amino acids indispensable for the catalytic activity of horse liver ADH (3) are highly conserved in the five ADHs (Fig. 4). The catalytic zinc atom of horse liver ADH is bound by three protein ligands, one sulfur atom each from Cys-38 (cysteine at position 38 of ADH-T) and Cys-148 and one nitrogen atom from His-61. These amino acids are completely conserved. The ligands of the second zinc atom, Cys-92, Cys-95, Cys-98, and Cys-106, are also conserved. Furthermore, one of the amino acids participating in the proton release system, a serine residue of horse liver ADH corresponding to position 40 of ADH-T, is substituted with threonine in other ADHs, including ADH-T. Serine and threonine (at position 40 of ADH-T) play the same function through their hydroxyl group. Another amino acid in the proton release system, His-43, is conserved, except in maize ADH. Thr-152, which has been reported to be important for proper positioning of NAD (18), is strictly conserved (Fig. 4).

**A putative reaction mechanism for ADH-T.** According to the argument mentioned above, it is believed that these ADHs, including ADH-T, have the same reaction mecha-

nisms as that shown for horse liver ADH (3). The basic reaction mechanism of horse liver ADH is as follows. One equivalent of proton is released per equivalent of ethanol that is oxidized. This proton release is associated with NAD binding and is dissociated from the water molecule bound to a catalytic zinc. This proton release from the water molecule occurs via the hydrogen bond system through the side chain (hydroxyl group) of Ser-40 (horse liver ADH in Fig. 4) to the imidazole ring of His-43. The proton is released at the surface of the molecule and not into the interior of the substrate-binding pocket. Then alcohol binds to zinc as the alcoholate ion, displacing the hydroxyl ion. The zinc atom polarizes the alcoholate so that direct hydrogen transfer and subsequent rearrangement to aldehyde can occur (3, 9). A putative proton release system for ADH-T was predicted by following the mechanism of horse liver ADH and is diagrammed in Fig. 5.

**Analysis of the proton release system by amino acid substitution.** To verify the reaction mechanism of ADH-T, the putative catalytic amino acid residues, Cys-38, Thr-40, and His-43, were substituted by site-directed mutagenesis with chemically synthesized oligonucleotides (Fig. 6). The following mutant enzymes were produced: Cys-38-Ser (Cys-38 as a putative catalytic zinc ligand was replaced by serine), Thr-40-Ala, Thr-40-Ser, and His-43-Ala. Their cell lysates were used for ADH assay and SDS-PAGE. All mutant enzymes were produced at the same level (data not shown). However, Cys-38-Ser, Thr-40-Ala, and His-43-Ala had no ADH activity. In contrast, Thr-40-Ser showed ADH activity. The wild-type ADH-T and the mutant enzyme Thr-40-Ser were purified to homogeneity. Thr-40-Ser had the same pH profile as the wild-type enzyme, although the level of enzyme activity was lower (Fig. 7). These results indicate that Cys-38, His-43, and the hydroxyl group of Thr-40 or Ser-40 are essential for enzyme activity and that the lower



**FIG. 5. Putative mechanism for the proton release system for ADH-T. This system is composed of a zinc-bound water molecule, Thr-40, and His-43, and the proton release is induced by NAD binding.**

level of activity of Thr-40-Ser might be explained by a subtle change of steric conformation. The proton in the ADH-T reaction mechanism would be transported from a water molecule, through the hydroxyl group of Thr-40, and released from the imidazole ring of His-43 (Fig. 5).

**Alteration of the enzyme pH profile by site-directed mutagenesis.** His-43 was substituted by arginine to alter the  $pK_a$  of the side chain (i.e., the  $pK_a$  of the imidazole ring of histidine is 6.0 and that of the guanidino group of arginine is 12.5). We inferred that this mutation might disturb the  $pK_a$  of the proton release group and result in a pH dependence different from that of wild-type ADH-T. The mutant enzyme His-43-Arg was produced and purified to homogeneity. The pH dependences of ADH-T, Thr-40-Ser, and His-43-Arg were tested by using purified enzymes. Wild-type ADH-T and Thr-40-Ser showed maximum activity at around pH 7.8, corresponding to the  $pK_a$  of 7.6 of the proton release group of horse liver ADH in the presence of NAD. In contrast, His-43-Arg exhibited a lower level of activity under acidic conditions but a higher level of activity under alkaline conditions than the wild type did. The maximum activity was observed at pH 9.0. Surprisingly, the maximum activity of His-43-Arg was about twofold higher than that of the wild type (Fig. 7). Thus, the optimum pH of ADH-T was shifted from neutral to alkaline by replacing the catalytic amino acid His-43 with arginine.

## DISCUSSION

The ADH gene (*adhT*) from *B. stearotheophilus* was cloned in *B. subtilis*. Wild-type ADH-T and its derivatives were easily purified from the transformants to homogeneity by heat treatment and DEAE-cellulose ion-exchange chromatography. Heat treatment is a powerful step to purify thermostable enzymes as shown in Fig. 1.

By site-directed mutagenesis, some *adhT* mutants were constructed. Studies with the mutant enzymes, which were constructed on the basis of three-dimensional-structure information available for horse liver ADH, provided considerable information about ADH-T catalysis. Thr-40 and His-43 should be essential as the active center of ADH-T. Cys-38 would be a ligand of the catalytic zinc. Enzyme catalysis for ADH-T would occur by the proton release system (Fig. 5). The pH profile of ADH-T was altered by replacing the catalytic amino acid histidine, His-43, with arginine. By the substitution, the  $pK_a$  of the active group, which was composed of a water molecule, Thr-40, and His-43, was thought to be shifted from neutral to alkaline. As a result, the alkaline enzyme His-43-Arg was obtained. Under acidic conditions, the mutant enzyme exhibited a lower level of activity than

|           |    |                                                           |    |
|-----------|----|-----------------------------------------------------------|----|
| Wild type | 5' | TGT GGG GTA TGC CAT ACA GAC TTG CAT GCC GCA CAT GGC GAC   | 3' |
|           |    | 35                        40                        45    |    |
|           |    | -Cys-Gly-Val-Cys-His-Thr-Asp-Leu-His-Ala-Ala-His-Gly-Asp- |    |
| Cys38Ser  |    | GT GGG GTA TCC CAT ACA GAC                                |    |
|           |    | -Ser-                                                     |    |
| Thr40Ala  |    | GTA TGC CAT GCA GAC TTG C                                 |    |
|           |    | -Ala-                                                     |    |
| Thr40Ser  |    | GTA TGC CAT TCA GAC TTG C                                 |    |
|           |    | -Ser-                                                     |    |
| His43Ala  |    | GC CAT ACA GAC TTG GCT GCC GCA CAT GGC G                  |    |
|           |    | -Ala-                                                     |    |
| His43Arg  |    | CA GAC TTG CGT GCC GCA C                                  |    |
|           |    | -Arg-                                                     |    |

FIG. 6. Nucleotide sequence and deduced amino acid sequence of the catalytic site and its flanking regions. Synthetic oligonucleotides to introduce mutations are shown. Mutated amino acids are indicated below the nucleotides. Amino acid numbers are shown above the amino acid sequences.

did wild-type ADH-T. The explanation for this might be that substitution of His-43 with arginine slowed down the proton release reaction under acidic conditions.

Generally speaking, the  $pK_a$  value of the active center of an enzyme can influence the pH profile. In other words, the pH profile of an enzyme could be altered by changing the  $pK_a$  value of a catalytic amino acid. For example, an active-site histidine residue of serine protease acts as a general base in enzyme catalysis, and its  $pK_a$  rules enzyme activity. Increasing the overall negative charge on the enzyme should raise the  $pK_a$  of the active-site histidine by stabilizing the protonated form of the histidine, whereas increasing the positive charge should lower the  $pK_a$  by

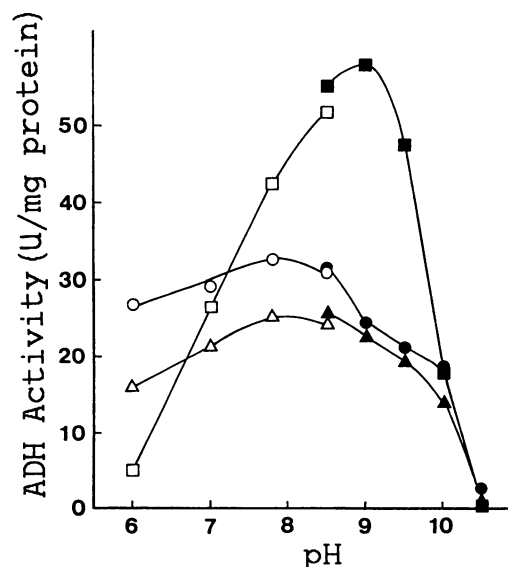


FIG. 7. pH profiles for ethanol of wild-type ADH-T (○ and ●), Thr-40-Ser mutant enzyme (△ and ▲), and His-43-Arg (□ and ■). Open symbols, enzyme assay in 100 mM potassium phosphate buffer; closed symbols, enzyme assay in 100 mM glycine-KOH buffer. Enzyme activity was assayed under the standard conditions described in the text, except for buffer pH. When the NAD concentration in the reaction mixtures was reduced to 0.2 mM, nearly the same results were obtained. Therefore, 1.0 mM NAD was actually excess at different pH conditions.

destabilizing the protonated form of the histidine. Its activity under acidic condition increased when the number of lysine residues of the enzyme surface was increased by site-directed mutagenesis (28).

Since enzymes are proteins containing many ionizable groups, they exist in a whole series of different states of ionization. However, only one of the ionic forms of the active center is catalytically active (7, 23). Our experiment shows that the  $pK_a$  value of an active site is responsible for the pH profile of an enzyme and that the optimum pH is altered by substituting a catalytic amino acid.

The activity of the His-43-Arg mutant enzyme at its optimum pH of 9.0 is about twice that of the wild type at pH 7.8. Arg-43 rather than His-43 might be more sterically suitable for proton transfer from Thr-40. The level of ADH activity of Thr-40-Ser at its optimum pH of 7.8 is lower than that of the wild type, perhaps because of steric hindrance. Crystallographic analysis of ADH-T is in progress.

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