interesting to determine the substrate activity of $ADPSO_3$ with this enzyme.

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Site-Directed Alteration of Four Active-Site Residues of a Pyruvoyl-Dependent Histidine Decarboxylase†

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ABSTRACT: Site-directed mutagenesis has been used to examine the chemical roles of four active-site residues in histidine decarboxylase (HDC) from *Lactobacillus* 30a. This protein is known to undergo an autoactivation in which chain cleavage between serines-81 and -82 leads to cofactor (pyruvoyl) formation at position 82. Conversion of Ser-81 to Ala virtually eliminates productive cleavage. It is proposed that the residue plays a key role in stabilizing the transition state of the chain cleavage reaction. Conversion of Phe-83 to Met renders the proenzyme thermally less stable than wild type and appears to slightly increase the rate of autoactivation. The K_m value for histidine is increased about 8-fold, confirming crystallographic evidence that Phe-83 is involved in substrate binding. Both wild-type and F83M enzymes show constant K_m and steadily increasing k_{cat} values as a function of temperature. Lys-155 and Tyr-262, by virtue of their positions in the active site of HDC, have been proposed to possibly play specific roles in either autoactivation or catalysis by active HDC. Conversion to Gln and Phe respectively suggests that these residues have real but minor roles in those processes.

Lactobacillus 30a, isolated from equine stomach, can be induced by histidine to express the gene for prohistidine decarboxylase (proHDC). The 310-residue proenzyme, or π chain, is then activated by chain cleavage between Ser-81 and Ser-82, creating an 81-residue β chain and the larger α chain. This process involves attack on the carbonyl of Ser-81 by the side chain of Ser-82. The intermediate undergoes β elimination to form a Schiff base which is ultimately hydrolyzed to produce a pyruvoyl moiety at the amino terminus of the α chain (Recsei

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et al., 1983). This group serves as the enzyme cofactor for conversion of histidine to histamine and CO_2. The mechanism of action has been shown to involve Schiff base formation between the substrate and pyruvoyl group (Recsei & Snell, 1970). Surprisingly, the pyruvoyl-dependent histidine decarboxylase has been shown to be as enzymatically efficient as the pyridoxal phosphate requiring enzyme from *Morganella*, even though the pyridoxal group is a far better electron sink than pyruvate (Abell & O'Leary, 1988). For a comprehensive review of the biochemistry of this enzyme class, see van Poelje and Snell (1990).

Because of its unusual biochemistry and activation, HDC has been the subject of considerable study over the years (van Poelje & Snell, 1990). The X-ray structure has been solved to 2.8-Å resolution (Parks et al., 1985). It shows HDC molecules are arranged as a sturdy trimer with a deep central hole containing three active sites; two trimers can also form weak tail to tail interactions to form a hexamer (Hackert et al., 1981). The X-ray structure also allowed identification of a number of amino acid groups in the activation/active-site area (Hackert et al., 1987; Gallagher et al., 1989). We have cloned and sequenced the gene which we called *hdc*A (Vanderslice et al., 1986; Copeland et al., 1989). The gene has been expressed in *Escherichia coli* and the resulting protein purified and characterized (Copeland et al., 1987).

Site-directed mutagenesis was used to explore the activation process by converting the key Ser-82 to both a Cys (S82C) and a Thr (S82T) (Vanderslice et al., 1988). This study showed that an intermediate, probably an ester, was formed during activation because ^{35}S label from position 82 of S82C could be seen to migrate to the β chain during productive cleavage. In both mutants, however, a side reaction occurred which caused nonproductive chain cleavage, that is, chain scission without cofactor production. This phenomenon is consistent with the ester intermediate being hydrolyzed to simply split the chain.

A second mutational study examined the role of carboxylate residues Glu-197 and Glu-66. Site-specific alteration showed Glu-197 is a key residue in enzyme catalysis and donates a proton to the Schiff base intermediate. Conversion to Gln reduced the catalytic rate constant 8000-fold. Its role as proton donor was confirmed by the conversion to Asp, which caused the enzyme to undergo a suicide reaction triggered by protonation of the catalytic complex at the wrong carbon. Conversion of Glu-66 to Gln showed it is part of a system governing the cooperativity of the enzyme kinetics, and mutation at this residue altered the Hill number for the reaction (McElroy & Robertus, 1989).

Figure 1 shows a schematic drawing of several residues in the active site of HDC. It is based on the X-ray model of the activated protein and on the binding of substrate analogues (Parks et al., 1985; Hackert et al., 1987; Gallagher et al., 1989). The drawing shows histidine in a Schiff base linkage to the pyruvoyl moiety at position 82. Glu-197, known to protonate an intermediate (McElroy & Robertus, 1989), is shown in its position near the substrate carboxylate. In addition, four other HDC residues are shown to indicate the rationale for their mutagenesis in this study. Ser-81 is the C terminus of the β chain of HDC; its carboxylate ion pairs to the substrate imidazolium ion. Phe-83 serves as the floor of the substrate binding site (Gallagher et al., 1989), and its ring is roughly perpendicular to the imidazole ring. It is likely that this residue is in a slightly different position in proHDC, when the neighboring Ser-82 is covalently linked to Ser-81. The figure also shows that the carbonyl of Phe-83 appears to hydrogen bond to the side chain of Tyr-262 in HDC. Tyr-262 may play a different role in proHDC where the position of Phe-83 is almost certainly perturbed. Finally, Lys-155 projects into the active-site cavity in the vicinity of the vital Glu-197. Lys-155 has been proposed as a candidate for specific catalytic tasks in either autoactivation or catalysis (Hackert et al., 1987).

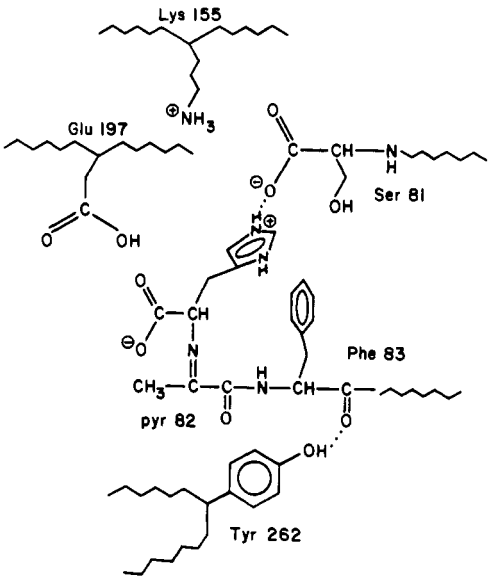

FIGURE 1: Schematic drawing of the active-site region of HDC. The histidine substrate (dark bonds) is shown in a Schiff base linkage to the pyruvoyl cofactor. The positions of certain HDC residues with respect to the substrate are indicated.

In this paper, we use site-directed mutagenesis to analyze the roles of these residues in either autoactivation or enzyme action. In each case, conservative changes were made to expose specific mechanistic attributes the residues might possess. In particular, Ser-81 was converted to Ala (S81A), Phe-83 to Met (F83M), Lys-155 to Gln (K155Q), and Tyr-262 to Phe (Y262F).

Materials and Methods

Most of the nucleic acid and protein methods used in this study have been described previously (Copeland et al., 1987; Vanderslice et al., 1988). However, site-directed mutagenesis was performed using uracil-containing template produced by the method of Kunkel (1985). An *E. coli dut⁻ ung⁻* strain, CJ236 (Kunkel et al., 1987), was infected with an *hdc*-containing M13 phage, and ssDNA-containing uracil was isolated as before (Copeland et al., 1987). Mutagenic oligonucleotide primers were typically 22 bases in length and contained 2 mismatches near the center which will produce the desired amino acid substitution. Unlike our previous work, the primers were extended by using a variation of the method of Craik et al. (1985). That is, primers were extended by using T4 DNA polymerase (Boehringer Mannheim) in the presence of 100 μg/mL T4 gene product 32 (Boehringer Mannheim), a ssDNA binding protein. T4 DNA ligase was excluded from the reactions, however, since we have observed some problems with deletion of inserted DNA in the presence of ligase. The uracil-containing dsDNA was transformed into a *dut⁺ ung⁺* strain, usually JM105, where RF replication greatly favors the mutant strand over the uracil-containing wild-type strand. Subsequent work, including gene sequencing and transfer to pUC plasmids for protein expression, was identical with that reported previously.

Protein was isolated as described previously (Copeland et al., 1987), except for the proF83M protein which was ther-

mally less stable than either wild type or the other mutant proteins isolated to date. *E. coli* DH5α cells were obtained from Bethesda Research Laboratories. Their genotype is F$^-$, *end*A1, *hsd*R17 (r_k^-,m_k^+), *sup*E44, *thi*-1, –, *rec*A1, *gyr*A96, *rel*A1, (*lac*ZYA-*arg*F)U169, Φ80d*lac*Z M15 (Jessee, 1986). Cells were transformed by standard methods (Ausbel et al., 1987), and transformants containing the F83M recombinant plasmid were grown at 30 °C overnight in 2 L of 2× YT medium (Miller, 1972) supplemented with 80 μg/mL ampicillin. Cells were harvested, and protein was purified according to the usual protocol with the following modifications. To prevent protein precipitation, the 68 °C heat treatment was replaced by one at 45 °C, which left the protein less pure than normal. Following the usual Sephacryl-200 molecular sieve column, the peak fractions were pooled and dialyzed against 0.02 M NH_4OAc, pH 6.5. The protein was loaded onto a 100-mL Q Sepharose (Pharmacia) column equilibrated in the same buffer, and 60 4-min fractions were collected at a flow rate of 1 mL/min. The protein was then eluted with a 500-mL linear gradient of 0–0.5 M NaCl in the same buffer. The peak OD_{280} fractions were checked for purity by SDS–PAGE. The pure fractions were pooled, dialyzed against 0.2 M NH_4OAc, pH 4.8, concentrated, and then used for further characterization studies.

The stability of the F83M protein was compared to wild type as a function of temperature; 60-μg samples (0.1 mL) of the proenzyme and activated forms of wild-type and F83M proteins were heated in a water bath at each of the following temperatures: 37, 45, 50, 55, 60, and 65 °C. After 10 min, each solution was cooled under running water for 10 s and placed on ice 10 min, and precipitated protein was removed by centrifugation. Three microliters of each sample supernatant was added per lane on a 10.5% SDS–polyacrylamide gel and electrophoresed. The bands were stained with Coomassie Blue and scanned by using an EC910 densitometer. The peaks were cut out and weighed to quantify the remaining soluble protein.

Mutant proenzyme was analyzed to determine the half-time (τ) of the first-order autoactivation process using gel electrophoresis and densitometry to measure the rate of π-chain cleavage (Copeland et al., 1987). Phenylhydrazine titration of the pyruvoyl group was used to measure productive chain cleavage. Initial rate kinetic data were determined at pH 4.8 for histidine concentrations of 0.05–7.2 mM. V_{max} and K_m values were determined from double-reciprocal or Eadie–Hofstee plots. The best line was determined by least-squares linear regression analysis using the program Sigmaplot 4.0 (Jandel Scientific). V_{max} was divided by the concentration of cofactor to calculate k_{cat}.

Results and Discussion

Ser-81 to Ala (S81A). All studied pyruvoyl-containing histidine decarboxylases have the activation site sequence Ser-Ser-Phe, centered around the equivalent of Ser-82, which initiates the activation reaction (Huynh & Snell, 1985). Although Ser-81 is conserved, no role has been hypothesized for it in autoactivation. To assess its role, we converted Ser to Ala, in essence removing the side chain oxygen and the ability of the side chain to participate in hydrogen bonding. The mutation had a profound effect on τ, the half-time for chain cleavage and presumed autoactivation. The first-order rate was so slow it could only be estimated by extrapolation of limited data, which showed only 2% cleavage in 50 days. This corresponds to a τ value of 1500 days, a 7000-fold increase over the wild-type value of 4.9 h (Table I). Because the activation was so slow, it was impractical to produce sufficient cleaved material to carry out a meaningful kinetic analysis on the S81A protein. As will be described below, it appears that the chain cleavage is not accompanied by productive cofactor formation in this mutant.

Table I: Autoactivation and Catalytic Parameters for Various Histidine Decarboxylases[a]

protein	τ (h)	K_m (mM)	k_{cat} (min^{-1})
wild type	4.9	0.4	2800
S81A	3.6E4		
F83M	3.9	3.2	1300
K155Q	6.1	0.3	360
Y262F	14.5	0.2	500

[a] τ is the half-time for the first-order autoactivation in 0.8 M K^+, pH 7.6, at 37 °C. Kinetic parameters were determined at pH 4.8.

Attempts by the Hackert laboratory to crystallize the S81A protein in 3.2 M ammonium sulfate caused the protein to cleave more rapidly (Wong, 1989). Subsequent studies showed a π-cleavage half-time of 129 days under the very high salt conditions. The amino-terminal sequence of the apparent α chain revealed that the main, although not exclusive, site of cleavage was between residues 80 and 81, not between 81 and 82 (Wong, 1989). This led to the conclusion that the π chain is under steric strain which may contribute to the activation process, but still leaves unanswered the question of what chemical role the side chain of Ser-81 plays in the process. The mutant protein crystallizes isomorphously with the wild type. The crystals contain mostly uncleaved protein, and a 3-Å difference Fourier map has been produced. It shows a chain of density near the carboxyl terminus of the β chain which may represent the path of the proHDC backbone. There is also evidence for movement of groups such as Phe-83. However, no detailed fitting of the position of Ala-81 or Ser-82 or comprehensive model of the proenzyme could be made (Wong, 1989).

The magnitude of change in cleavage rate for S81A is surprising and suggests a major role for Ser-81. Conversion of the key Ser-82 to a Cys decreased the activation rate only 34-fold (Vanderslice et al., 1988). It seems unlikely that the structurally conservative change from Ser to Ala would have a major effect on the overall protein folding or structure, and indeed the difference Fourier confines the changes in the mutant to the immediate area of chain scission. Instead it seems reasonable to propose that Ser-81 plays a major catalytic role in the autoactivation process.

One possible role for the residue is based on the observation that the autoactivation process has an interesting feature in common with serine protease reactions, namely, that a peptide bond is attacked by a Ser residue and an ester intermediate is likely formed. During the protease attack, the carbonyl carbon shifts from trigonal to tetrahedral configuration, and the carbonyl oxygen can be formally thought of as an oxyanion. This structure resembles the transition state of the reaction and is largely stabilized by receiving specific hydrogen bonds from residues constituting an "oxyanion hole" (Robertus et al., 1972). The role of the binding site was proposed on the basis of X-ray structures and was later confirmed by site-directed mutagenesis studies (Wells et al., 1986; Bryan et al., 1986).

It seems plausible that a similar oxyanion hole exists in prohistidine decarboxylase or that some group must help stabilize the developing negative charge on the Ser-81 carbonyl oxygen during autoactivation. This may be the role for the required cation. However, because of its close proximity, it may be that the side chain of Ser-81 performs this stabilizing role by donating a hydrogen bond to the developing oxyanion.

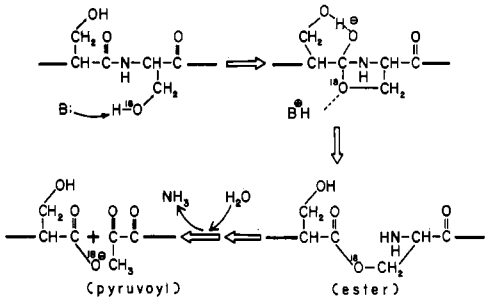

FIGURE 2: Possible role for Ser-81 in autoactivation of prohistidine decarboxylase. The OΓ of Ser-82 attacks the carbonyl carbon of Ser-81, changing its bonding geometry from trigonal to tetrahedral and causing a negative charge to develop as an oxyanion on the carbonyl oxygen. The rate of the reaction can be increased if the oxyanion intermediate can be stabilized by partial protonation. This is probably the role of the side chain hydroxyl of the conserved Ser-81. As shown, a six-membered ring can be formed to help stabilize the transition-state structure.

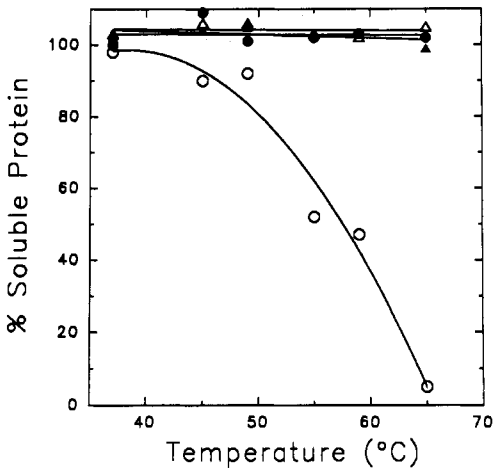

FIGURE 3: Thermal stability of wild-type and mutant HDCs. The percent of protein remaining soluble after a 10-min exposure to a range of temperatures is shown. The solid symbols are wild-type proteins, and the open symbols are F83M proteins. Circles are proenzyme forms, and triangles are activated enzymes.

This is illustrated in Figure 2. The S81A mutation may be dramatic in its effects because it directly alters a group involved with stabilization of the transition state of the autoactivation reaction.

Phe-83 to Met (F83M). The site of autoactivation of all known pyruvoyl-dependent HDCs has a Phe residue immediately downstream from the susceptible Ser cleavage site (Huynh & Snell, 1985). The difference Fourier for S81A discussed above also suggests that Phe-83 moves during autoactivation, and this rearrangement may play a key role in proper formation of the active enzyme. Furthermore, X-ray analysis shows that in the activated enzyme, the side chain of Phe-83 forms the solid floor of the binding pocket for the imidazolium ring of the histidine substrate (Gallagher et al., 1989). It is interesting to note that the ring of Phe-83 is perpendicular to the plane of the imidazole ring of the substrate and this orientation may be important for optimum substrate alignment in the binding site. To explore these possible roles, we made a conservative change to Met. This residue is nonpolar and has about the same volume as Phe, but lacks its planar aromatic structure.

As mentioned under Materials and Methods, the F83M protein was thermally less stable than wild type, and the heat denaturation step of the purification procedure had to be modified. The purified proenzyme was activated in the usual way and showed a half-time value of 3.9 h compared to 4.9 h for wild type (Table I). Kinetic analysis of F83M at 37 °C showed the K_m to be 3.2 mM, 8-fold higher than wild type, while V_{max} was reduced to half that of wild type.

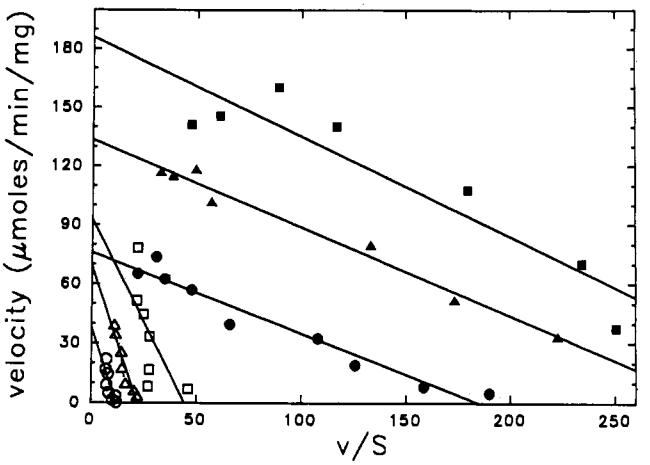

FIGURE 4: Effect of temperature on kinetic parameters. Eadie–Hofstee plots of initial rate data are show as a function of temperature. Solid symbols are wild-type HDC and open are F83M. Circles represent data at 37, triangles at 55, and squares at 65 °C.

Table II: Kinetic Parameters as a Function of Temperature[a]

protein	T (°C)	K_m (mM)	V_m	k_{cat}
wild type	37	0.41	80	2800
	55	0.45	133	4550
	65	0.50	186	6350
F83M	37	3.2	38	1300
	55	3.2	69	2350
	65	2.1	93	3200

[a] V_m is in micromoles per minute per milligram; k_{cat} is in moles per mole per minute.

To explore the observed temperature instability of the mutant protein, both proenzyme and activated forms of wild-type and mutant protein were exposed to a range of temperatures, and their solubilities were measured as described under Materials and Methods. As seen in Figure 3, only proF83M is thermally unstable. Once activated, F83M is as stable as wild type at temperatures up to 65 °C.

The stability of wild-type and F83M HDC was further examined by carrying out kinetic analyses at 37, 55, and 65 °C. The results are shown as Eadie–Hofstee plots in Figure 4. In these plots, the y intercept is V_{max}, and the negative slope is K_m. The figure shows that K_m for both enzymes is essentially independent of temperature, although the wild-type value is consistently 6–8 times lower than for F83M. V_{max}, and therefore k_{cat}, is a function of temperature for both proteins. The least-squares lines also show that k_{cat} is essentially twice as fast for wild type as F83M at all temperatures. These kinetic parameters are summarized in Table II.

The results of the above experiments suggest that proF83M is a relatively unstable protein, compared to wild type and to the other mutants we have made. Since the X-ray structure of the proenzyme is not yet solved, it is difficult to explain why the substitution of Met for Phe at this position should sensitize the protein to thermal denaturation. Once activated, F83M is stable, and so presumably activation allows the Met side chain to assume a new conformation which does not destabilize the structure. The rate of activation of the mutant proenzyme is slightly faster than for wild type. This may indicate that the high-energy conformation of the proenzyme, which makes it thermally unstable, helps drive the activation process. This

is consistent with the hypothesis that the proenzyme is under mechanical strain which predisposes it to the activation process (Wong, 1989).

Kinetic analysis shows that the mutation at Phe-83 primarily affects K_m, although k_{cat} is reduced 2-fold. To the extent that K_m is a rough measure of the affinity of the enzyme for substrate, Phe-83 appears to be involved in substrate binding. This is certainly consistent with the X-ray model which shows the side chain interacting with the imidazole of the substrate (Gallagher et al., 1989). The small but consistent effect on k_{cat} may arise because Met at position 83 causes a minor misalignment of the substrate in the active site, slowing the bond-breaking step.

Analysis of the kinetic parameters as a function of temperature shows that over the small range tested, K_m does not vary for either wild type or F83M. This suggests that the ability to recognize substrate is not being perturbed by thermal motion even up to 65 °C, a temperature which would denature many enzymes. It seems reasonable that the strong trimeric structure of HDC helps make it very stable. Temperature clearly affects k_{cat} for both mutant and wild-type enzymes. Catalytic rates rise smoothly with the increase in thermal energy. In both cases, k_{cat} increases by a factor $\approx$1.37 for each 10 °C rise in temperature. Again, there is no suggestion from these data that the protein has begun to unfold, even at 65 °C, which presumably would counteract the thermal energy and begin to diminish catalytic efficiency.

Lys-155 to Gln (K155Q). Lys-155 occupies a prominent position in the active-site cleft of HDC (Hackert et al., 1987; Gallagher et al., 1989). Because of its position, it has been proposed that Lys-155 might serve as the proton donor either in the autoactivation scheme or in the enzymatic reaction of histidine decarboxylase (Hackert et al., 1987). To test this hypothesis, we converted Lys-155 to Gln. Glutamine is a long polar residue, about the same size as lysine, which is able to donate and accept hydrogen bonds. Unlike lysine, however, it lacks a positive charge and is unable to act as a true acid. As shown in Table I, the mutation has very little effect on the autoactivation rate. Once activated, the K155Q protein has a K_m which is, within experimental error, identical with wild type. The catalytic rate constant, however, has decreased 8-fold. Although this change is small compared to that seen for many other mutants (Vanderslice et al., 1988; McElroy & Robertus, 1989), the effect is real. It seems unlikely, however, that Lys-155 is a specific proton donor in either autoactivation or catalysis. Gln cannot act as an acid, and its substitution in the enzyme at position 155 has too little effect to confirm that role for the normal Lys group. Instead, Lys-155 may play a more general role in maintaining the active-site geometry. It may act as a hydrogen bond donor and might even serve to orient solvent. This role may be mimicked reasonably well by glutamine. A clearer role may be defined by further X-ray analysis of the environment.

Tyr-262 to Phe (Y262F). On the basis of the X-ray structure, it was suggested that Tyr-262, which is found near the pyruvoyl group of the active enzyme, might perform one of several functions in the protein. It might, for example, serve to donate a hydrogen bond to the amide oxygen of the pyruvoyl group and thereby increase the electron-withdrawing activity of the cofactor with respect to the substrate (Hackert et al., 1987). It has also been observed that autoactivation is a function of pH and that the process appears to require a proton donor with a pK_a of 8.2 (Recsei & Snell, 1981). Although it was hypothesized that a Cys residue might play this role, the X-ray structure ruled out that possibility. The structure did suggest that Tyr-262, along with Lys-155 or Tyr-62, might act as the acid, although the pK_a of the group would have to be perturbed modestly (Hackert et al., 1987). To investigate the role of Tyr-262, we converted the residue to a Phe, in essence removing the hydroxyl function which can participate in hydrogen bonding or serve as an acid.

The mutation had only a modest effect on protein behavior (Table I). τ is increased 3-fold, and K_m is decreased by a factor of 2. The catalytic rate constant is decreased 6-fold, but again this is not very substantial. In fact, k_{cat}/K_m, the specificity constant (Fersht, 1985), is only 3-fold lower for Y262F than for the wild type, indicating that the mutation has had little effect on the overall activity of histidine decarboxylase. It seems unlikely, therefore, that Tyr-262 plays any major specific role in enzyme action, although it may help stabilize the optimum conformation for the enzyme.

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Registry No. HDC, 9024-61-7; L-Ser, 56-45-1; L-Phe, 63-91-2; L-Lys, 56-87-1; L-Tyr, 60-18-4; L-His, 71-00-1.

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General Base Catalysis in a Glutamine for Histidine Mutant at Position 51 of Human Liver Alcohol Dehydrogenase†

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ABSTRACT: On the basis of the three-dimensional structure of horse liver alcohol dehydrogenase determined by X-ray crystallography, His 51 has been proposed to act as a general base during catalysis by abstracting a proton from the alcohol substrate. A hydrogen-bonding system (proton relay system) connecting the alcohol substrate and His 51 has been proposed to mediate proton transfer. We have mutated His 51 to Gln in the homologous human liver $\beta_1\beta_1$ alcohol dehydrogenase isoenzyme which is expected to have a similar proton relay system. The mutation resulted in an about 6-fold drop in V/K_b (V_{max} for ethanol oxidation divided by K_m for ethanol) at pH 7.0 and a 12-fold drop at pH 6.5. V/K_b could be restored completely or partially by the presence of high concentrations of glycylglycine, glycine, and phosphate buffers. A Brønsted plot of the effect on V/K_b versus the pK_a of these bases plus H_2O and OH^- was linear. Only secondary or tertiary amine buffers differed from linearity, presumably due to steric hindrance. These results suggest that His 51 acts as a general base catalyst during alcohol oxidation in the wild-type enzyme and can be functionally replaced in the mutant enzyme by general base catalysts present in the solvent. Steady-state kinetic constants for NAD^+ and the trifluoroethanol inhibition patterns were similar between the wild-type and the mutant enzyme. Differences in the inhibition constants (K_i) of caprate and trifluoroethanol below pH 7.8 and in the pH dependence of K_i can be explained by the substitution of neutral Gln for positively charged His.

Enhancement of chemical reactions by enzymes in many cases involves acid/base catalysis by functional groups in the enzyme. The imidazole side chain of histidine with a pK_a value around 7 is a particularly suitable acid/base catalyst (proton donor/acceptor) at pH 7; e.g., a histidine located in the active center of papain, chymotrypsin, glyceraldehyde-3-phosphate dehydrogenase, and lactate dehydrogenase has been suggested to act as a proton donor/acceptor (Fersht, 1984). In the horse liver alcohol dehydrogenase EE isoenzyme, the imidazole ring of His 51 is about 6 Å from the enzyme-bound alcohol substrate hydroxyl group. This distance precludes direct proton transfer, but the three-dimensional structure suggests that alcohol and His 51 are connected by a hydrogen-bonding system involving hydroxyl groups of Ser 48 and of the coenzyme nicotinamide ribose (Eklund et al., 1982). This hydrogen-bonding system has been proposed to mediate proton transfer and has been named the "proton relay system" (Eklund et al., 1982).

The EE isoenzyme of horse liver ADH[1] is a member of a family of mammalian isoenzymes termed class I ADH (Vallee & Bazzone, 1983). It is a dimer and contains two atoms of Zn^{2+} per subunit (Eklund et al., 1976). The horse liver EE isoenzyme shares 88% amino acid sequence identity with the human liver $\beta_1\beta_1$ isoenzyme (Hempel et al., 1984). Importantly, $\beta_1\beta_1$ contains the same active site residues as EE except for Ser 48 and Ile 318, which are conservatively replaced by Thr and Val, respectively, in $\beta_1\beta_1$ (Eklund et al., 1987). Computer modeling studies suggest that the hydroxyl of Thr 48 in $\beta_1\beta_1$ can form the same hydrogen bonds as Ser in the EE isoenzyme (Eklund et al., 1987). It is therefore probable that the proton relay system exists in $\beta_1\beta_1$.

We have mutated His 51 in the $\beta_1\beta_1$ isoenzyme to Gln and examined the steady-state kinetics with ethanol and NAD^+,

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[1] Abbreviations: ADH, alcohol dehydrogenase; Cap-Gapp, 4-[3-[*N*-(6-aminocaproyl)amino]propyl]pyrazole; ACES, 2-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid; TAPSO, 3-[*N*-[tris(hydroxymethyl)methyl]amino]-2-hydroxypropanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate.

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