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

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ABSTRACT: The active site of histidine decarboxylase (HDC) from *Lactobacillus* 30a contains a pyruvoyl cofactor sitting at the interface of two molecules in a trimer. Although exhibiting hyperbolic kinetics at pH 4.8, near its optimum, HDC is cooperative at pH 7.6, indicating that the units of the trimer communicate. A Hill plot analysis shows that HDC, at pH 7.6, can be described by a two-state model. The tense (T) state has an apparent K_m for histidine of 50 mM, while the relaxed (R) state has a K_m of 5 mM. To explore the catalytic mechanism, three of the cross-boundary active-site residues were altered by site-directed mutagenesis and their effects observed. Ile-59 is known to act as lid on the substrate binding pocket; it was converted to Ala (I59A) and to Val (I59V). The former was inactive, attesting to the importance of this residue in the mechanism. The I59V mutant showed a decrease in K_m and in k_{cat} at pHs 4.8 and 7.6. Ile-59 appears to help orient substrate properly for catalysis; decreasing its size expands the binding site. This may allow the substrate to bind more readily, but in a number of conformations which are not optimal for catalysis. Conversion of Tyr-62 to Phe (Y62F) had no effect on catalysis but raised the K_m 7-fold at pH 4.8. Asp-63 appears to form an ion pair to the substrate imidazolium. Conversion to the neutral amide (D63N) had no effect on the k_{cat}, but raised the K_m 240-fold at pH 4.8. This is consistent with the notion that the ion pair is up to 3 kcal/mol stronger than a simple hydrogen bond with the substrate. The mutant had no detectable activity at pH 7.6.

Lactobacillus 30a, induced by histidine, expresses prohistidine decarboxylase (proHDC). This 310-residue proenzyme (π chain) is activated by a serinolysis reaction which cleaves the peptide chain between Ser-81 and Ser-82, creating an 81-residue β chain and a larger α chain. This process produces a pyruvoyl moiety at the amino terminus of the α chain which serves as the enzyme cofactor for conversion of histidine to histamine and CO_2. The mechanism of decarboxylation involves Schiff base formation between the substrate and pyruvoyl group (Recsei & Snell, 1970). The history and basic biochemistry of this enzyme have been exhaustively reviewed by van Poelje and Snell (1990).

The X-ray structure of activated histidine decarboxylase (HDC) has been solved to 3.0-Å resolution (Parks et al., 1985) and refined to 2.5-Å resolution (Gallagher et al., 1989). It shows that three HDC molecules trimerize, forming a central well with three active sites near the bottom. Two trimers can also form weak tail-to-tail interactions to form a hexamer (Hackert et al., 1981), but it is unlikely that this has any catalytic significance. The crystallographic model has allowed identification of a number of putative active-site/activation-site residues (Hackert et al., 1987; Gallagher et al., 1989). One important finding is that each active site is formed at the boundary of two molecules related by the molecular 3-fold axis. An active site formally located on molecule A will contain the pyruvoyl moiety and a number of amino acid groups such as Glu-197 and Lys-155 from that molecule. In addition, a number of residues from the β chain of neighboring molecule B will contribute to that active site. These include residues Ile-59, Tyr-62, Asp-63, and Glu-66 which we will refer to as "cross boundary" residues. In fact, all of these residues lie along one face of an α-helix, and their activities may be coordinated.

We have cloned and sequenced the gene for proHDC (Vanderslice et al., 1986) which is now called *hdcA* (Copeland et al., 1989). The gene was moved behind the β-galactosidase promoter of a pUC plasmid for expression in *Escherichia coli*; the resulting protein has been purified and characterized (Copeland et al., 1987). Site-directed mutagenesis has been used to explore the roles of a number of residues both in autoactivation and in catalysis (Vanderslice et al., 1988; McElroy & Robertus, 1989; Gelfman et al., 1991). For example, conversion of Glu-197 to Gln reduced the k_{cat} nearly 8000-fold. Conversion to Asp created a suicide enzyme due to misprotonation of the covalent catalytic intermediate. This confirmed the notion that Glu-197 functions as a specific acid in the wild-type catalytic mechanism (McElroy & Robertus, 1989).

It is also known that while HDC shows hyperbolic kinetics (Hill number of 1.0) at pH 4.8, near its optimum, it is a cooperative enzyme with sigmoidal kinetics at pH values over 7, with a Hill number of 2.0 at pH 7.6 (Recsei & Snell, 1970). Conversion of the cross-boundary residue Glu-66 to Gln (E66Q) results in a protein which exhibits cooperative kinetics, with a Hill number of 1.5, at both pH 4.8 and pH 7.5 (McElroy & Robertus, 1989). The fact that E66Q shows distinctly sigmoidal kinetics even at pH 4.8 was discussed in terms of the usual two-state models for cooperative enzymes (McElroy & Robertus, 1989). That is, HDC has a tense (T) form with poor substrate binding, which is in equilibrium with a relaxed, or R, state which binds substrate readily. The R state is favored by low pH, but at high pH, histidine acting as a homotropic effector also favors the R state. Under given conditions, HDC may be thought of as a linear combination of these states. The X-ray model for HDC was obtained at pH 4.8 (Hackert et al., 1981) and therefore is likely to reflect the R state of the enzyme.

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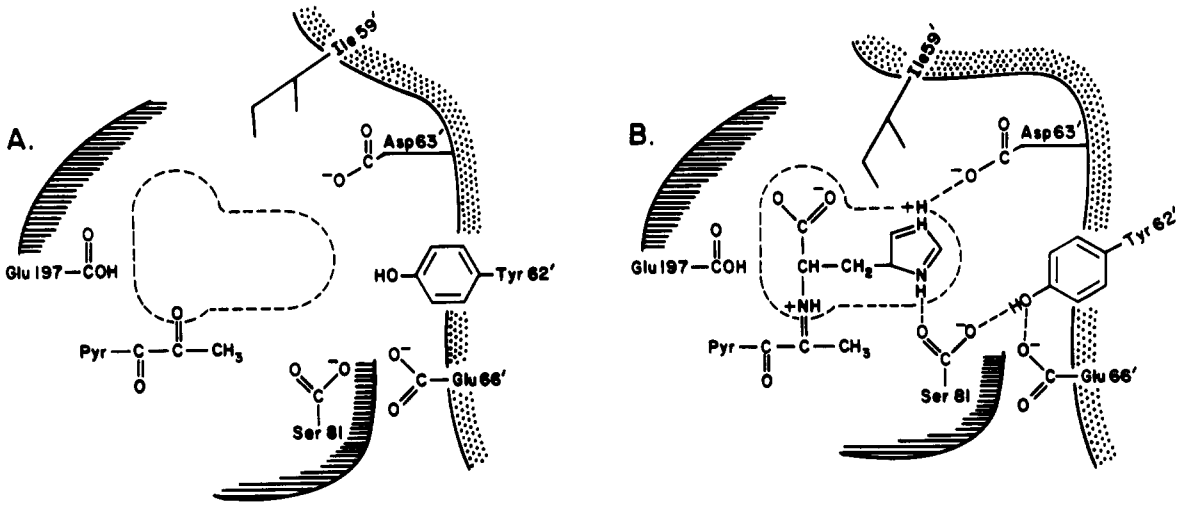

FIGURE 1: Schematic drawing of the active site of histidine decarboxylase. The drawing is based on the crystallographic results of Gallagher et al. (1989). Panel A shows the pyruvoyl-containing active site without substrate; the dashed enclosure is the histidine binding site. Ser-81 is the carboxy terminal of the β chain. Residues Ile-59′, Tyr-62′, Asp-63′, and Glu-66′ are cross-boundary residues contributed to the active site by an adjacent, 3-fold-related molecule. The boundaries of the separate molecules are indicated by the shaded curves. Panel B shows that histidine is bound via a Schiff base linkage to the pyruvate. Ile-59′ moves to form the lid of the binding pocket while Tyr-62′ rotates to participate in a hydrogen-bonded network. Asp-63′ forms an ion pair directly with the substrate imidazolium.

The effect of the E66Q mutation on HDC kinetics suggested that cooperativity might be facilitated by the cross-boundary residues. The presence of substrate at active-site A might be communicated to B through these groups as they interacted with the substrate. The crystal structure of HDC with bound substrate analogs, such as histidine methyl ester, showed that the largest protein movements in response to analog binding do occur in this area. Tyr-62 swings 30°, moving its hydroxyl by 3.3 Å, to hydrogen bond both with the carboxyl terminus of the α chain (Ser-81) and with the carboxylate of Glu-66. The Ser-81 carboxylate itself moves about 1 Å toward the imidazolium of the histidine substrate and makes a hydrogen bond or ion pair with it. Ile-59 moves 1.5 Å toward the substrate and acts as a flexible lid to the imidazole binding site, the floor being formed largely by Phe-83. At the same time, the carboxylate of Asp-63 appears to form an ion pair with the substrate imidazolium (Gallagher et al., 1989). Figure 1 shows a schematic drawing of this region, calling attention to the positions of the active-site groups including the cross-boundary residues; it is based on X-ray analysis performed at pH 4.8 (Gallagher et al., 1989) where HDC is presumably in the R state. The motions in the as yet unobserved T state may differ, but our working hypothesis is that similar motions may be involved in driving ligand-bound HDC toward the R form.

Since only Glu-66 of the cross-boundary residues had been probed by site-directed mutagenesis, we decided to make chemically conservative changes at three other apparently important positions. In particular, Ile-59 was converted to Ala (I59A), and to Val (I59V). Tyr-62 was converted to Phe (Y62F) and Asp-63 to Asn (D63N).

MATERIALS AND METHODS

Site-directed mutagensis was carried out as described in Gelfman et al. (1991). Briefly, uracil-containing template was produced by the method of Kunkel (1985). Mutagenic primers 29–33 bases in length and containing the codon for the desired alteration were extended using a variation of the method of Craik et al. (1985). The uracil-containing dsDNA was transformed into a *dut*$^+$ *ung*$^+$ strain, usually *E. coli* JM105 or DH5αF′, where RF replication greatly favors the mutant strand over the uracil-containing wild-type strands. Recombinant clones were screened for mutations by DNA sequencing using the appropriate primer from a panel of primers. Potential mutant clones were then sequenced over the length of the gene to be sure only the desired changes had been made. The mutant gene was transferred to a pUC-based plasmid for expression. Protein was isolated as described previously (Copeland et al., 1987).

Mutant enzymes were chemically characterized as described by Gelfman et al. (1991). Briefly, proenzyme autoactivation was followed using gel electrophoresis and densitometry to measure the half-time (τ) of the first-order 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 as previously described. In addition, kinetics were carried out at higher pH values to test for cooperative behavior. Histidine concentrations ranged from 0.05 to 800 mM, depending on protein activity and pH. Whenever possible, substrate levels spanned from roughly $0.1K_m$ to $10K_m$. For hyperbolic kinetics, V_m and K_m values were determined from double-reciprocal plots and also by least-squares nonlinear regression analysis using the program Sigmaplot 5.0 (Jandel Scientific); the methods gave comparable results. V_m was divided by the concentration of cofactor to calculate k_{cat}. For sigmoidal kinetics, $S_{0.5}$ and V_m were determined from nonlinear analysis of the initial rate data using Sigmaplot.

RESULTS AND DISCUSSION

Wild-Type Kinetics. In order to investigate the behavior of the cross-boundary mutants, it is necessary to have parameters for wild-type HDC which reflect our assay system. Recsei and Snell (1970) have carried out a characterization using a similar but not identical system.

Figure 2 shows initial rate data for HDC under a range of pH values, from 4.8 through 7.6. Figure 2a shows data for pH values from 4.8 through 6.5, while Figure 2b, using a substantially larger substrate range, shows data for pHs 7 through 7.6. The hyperbolic curves shown by data for 4.8 through 7.0 suggest that HDC is not acting cooperatively at these pH values, while the velocity profile at pH 7.6 is clearly sigmoidal. The kinetic parameters derived from these data are listed in Table I.

In Figure 3, the parameters have been plotted to display log

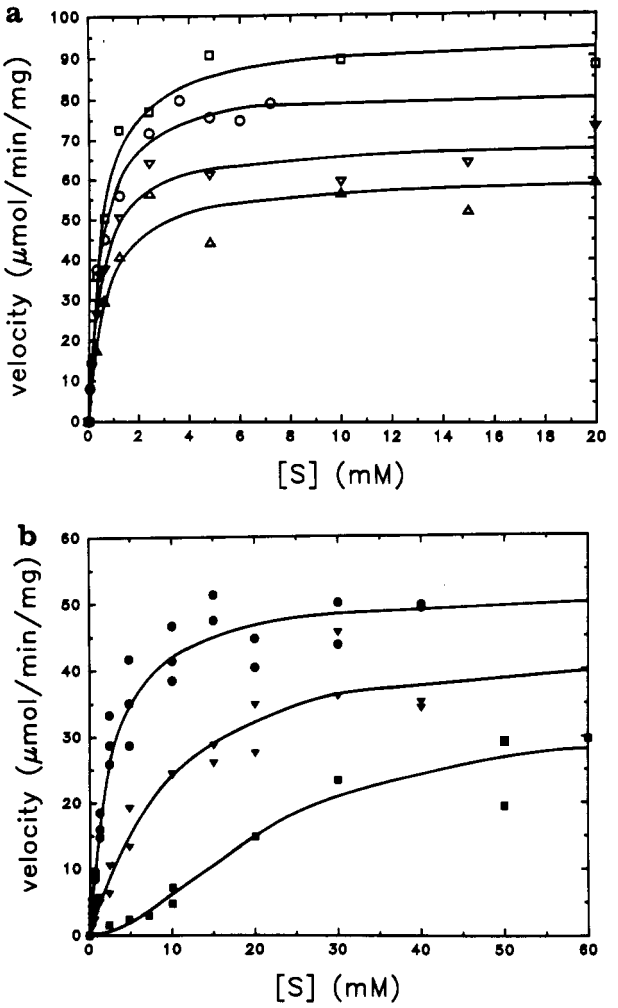

FIGURE 2: Initial velocity data for wild-type HDC as a function of pH. (a) Velocity plots for pH values of 4.8 (open circles), 5.5 (open squares), 6.0 (open downward triangles), and 6.5 (open upward triangles). (b) Velocity plots for pH values of 7.0 (solid circles), 7.2 (solid downward triangles), and 7.6 (solid squares).

Table I: Kinetic Parameters for Wild-Type HDC as a Function of pH

pH	V_m	k_{cat} (min^{-1})	K_m or $S_{0.5}$ (mM)	Hill number
4.8	83.8	2850	0.46	1.0
5.5	94.0	3200	0.46	1.0
6.0	68.8	2340	0.45	1.0
6.5	60.0	2040	0.61	1.0
7.0	51.9	1760	2.3	1.0
7.2	46.3	1570	9.1	1.2
7.6	32.0	1100	22	1.9

V_m, $-\log K_m$ (or $-\log S_{0.5}$), and the Hill number, as a function of pH. HDC has an optimum value for V_m, or k_{cat}, at pH 5.5; this is in agreement with Recsei and Snell (1970). As they pointed out, the relative flatness of this curve suggests that titratable groups do not play a major role in the rate-determining kinetic step.

The affinity for substrate, as estimated by the K_m for hyperbolic kinetics or the $S_{0.5}$ for cooperative data, is essentially constant over the pH range of 4.8–6.0 (Figure 3). The affinity then decreases rapidly, with a slope of –2. Again, this is consistent with the findings of Recsei and Snell (1970). The slope would generally be interpreted as meaning that substrate affinity is controlled by two groups with pK_a values around 6.5 (Dixon & Webb, 1964). Because HDC is changing from hyperbolic to sigmoidal kinetics, this interpretation may be

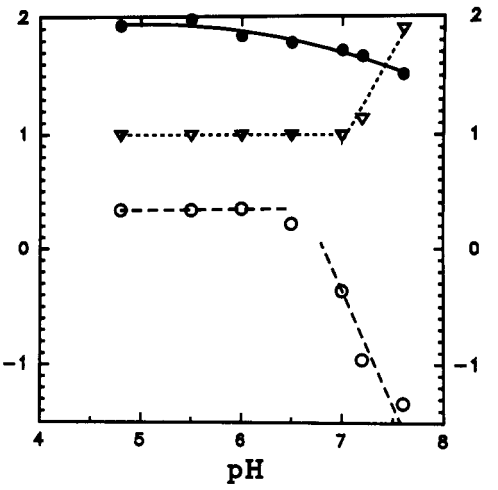

FIGURE 3: Kinetic parameters for wild-type HDC as a function of pH. Initial rate kinetic data from Figure 2 are replotted. The solid circles are log V_m, and the open circles are $-\log K_m$ or $-\log S_{0.5}$ as appropriate. The open triangles are the Hill numbers.

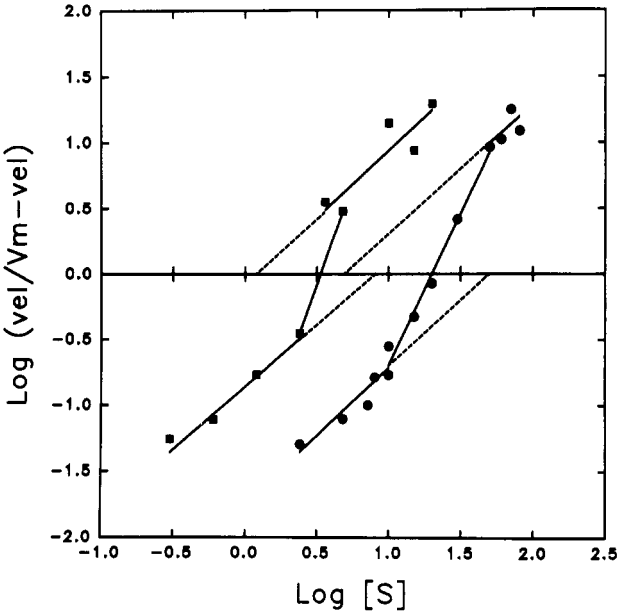

FIGURE 4: Hill plots of wild-type HDC and I59V at pH 7.6. Wild-type velocity data are shown as solid circles and those for I59V as solid squares.

suspect. Even so, it is reasonable to assume that two groups are involved and that one of these is the imidazolium of the substrate histidine; the uncharged form does not bind as well to HDC as does the charged form.

HDC at pH 7.6 shows a Hill number near 2; since the structural unit of enzymatic activity is undoubtedly a trimer, this reflects a relatively high level of cooperativity. A simple model for cooperative kinetics would propose that at low His concentration the HDC trimer is in a low substrate affinity T state and that increasing substrate concentration acts as a homotropic activator to drive the equilibrium toward the R state.

To analyze this further, the pH 7.6 kinetic data were replotted as shown in Figure 4. At His substrate concentrations from 0.3 to nearly 10 mM, HDC shows a Hill number of 1. This is consistent with the enzyme being in the T state and not acting cooperatively. Extrapolation of the velocity line to half of the maximal value suggests that the K_m for this T state, at pH 7.6, is roughly 50 mM. Between about 10 and 50 mM substrate levels, HDC shows a Hill number of 2 and

Table II: Autoactivation and Catalytic Parameters for Histidine Decarboxylase[a]

		pH 4.8			pH 7.6		
protein	τ (h)	K_m (mM)	k_{cat}/ min	k_{cat}/K_m ($\times 10^{-3}$)	$S_{0.5}$ (mM)	k_{cat}/ min	Hill number
wild type	5	0.4	2800	117	22	1100	1.9
I59A	11						
I59V	4	0.3	800	39	3	123	2.8
Y62F	17	2.8	2500	15	42	560	1.5
D63N	6	97	2600	0.5			

[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 pHs 4.8 and 7.6. K_m is not defined for cooperative kinetics; $S_{0.5}$ is the concentration of substrate yielding half-maximal velocity. The turnover number, k_{cat}, is calculated as V_m/[HDC]. k_{cat}/K_m is the apparent second-order rate constant and has units of s^{-1} M^{-1}.

an $S_{0.5}$ of 22 mM. His becomes inhibitory at pH 7.6 above about 100 mM, but the data between 50 and 80 mM show a slope (Hill number) of 1, indicating that HDC is in the R state. Extrapolation of the data on that limb of the graph suggests a K_m for the R state, at pH 7.6, of about 5 mM. At pH 7.6, the T-state K_m is 10 times larger than that for the R state.

As shown in Figures 2 and 3, decreasing the pH from 7.6 rapidly reduces the Hill number. Protons apparently act as heterotrophic effectors to stabilize HDC in a high-affinity form with hyperbolic kinetics and a K_m near 0.4 mM. It seems reasonable to suggest that this state resembles the high-affinity R form induced by His at pH 7.6 but shows an affinity for substrate which is at least 10-fold higher. This may reflect stronger interactions between HDC and the substrate at the more acidic pH, although more complicated phenomenon cannot be discounted. To help assess this, several site-directed mutations were made and analyzed.

Mutations at Ile-59. As indicated in Figure 1, Ile-59 moves about 1.5 Å upon substrate binding and acts as a lid on the imidazole binding pocket. The X-ray structures revealing the change in Ile-59 were derived at pH 4.8, when HDC is presumably in the R-state conformation even in the absence of substrate. It is unknown if the Ile-59 motion would occur in the T state. It is reasonable to suppose, however, that HDC in the T state would show similar changes upon substrate binding and that Ile-59 may therefore be involved in controlling HDC cooperativity. That is, information about a substrate molecule binding at active site A, even in the T state, could be transmitted to molecule B by the motion of Ile-59 interacting across the A/B boundary.

The first mutation made was the conversion of Ile-59 to Ala (I59A). This protein expressed well in *E. coli* and was characterized in the usual fashion. Table II lists activation and kinetic parameters for I59A as well as all the other mutants analyzed for this study. In analyzing the kinetic parameters of the mutants, it is important to recall that no X-ray structures are available for them and so conformational changes which might affect activity cannot be assessed. This is unlikely to be a major problem for the following reasons. Each mutant folds into hexamers indistinguishable from wild type during purification. Each mutant proenzyme autoactivates with a half-time near wild-type values, and so the active-site area must be intact. All but one activated mutant HDC exhibits kinetic parameters, K_m or k_{cat}, which are reasonably close to wild-type values. Again, this suggests that the mutant enzyme is catalyzing the decarboxylation reaction in the usual fashion and that major structural perturbation of active site is not a problem.

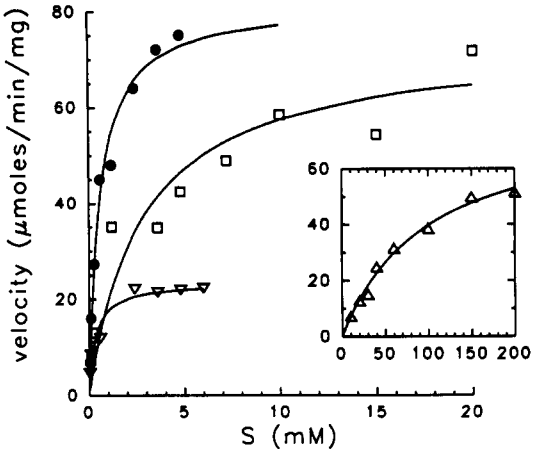

FIGURE 5: Initial velocity plots of wild-type and mutant HDCs at pH 4.8. The solid circles are wild type, the downward triangles are I59V, the squares are Y62F, and the triangles in the inset graph are for D63N. The axes on the inset have the same units as those of the main graph.

The activation of the I59A proenzyme proceeded normally. The half-time of autoactivation (τ) is only 2.2 times longer than for wild type, suggesting only a minor perturbation in the proenzyme structure. Titration with phenylhydrazine confirmed that pyruvoyl cofactor production was normal. The processed protein, however, was catalytically inactive. It exhibited no detectable decarboxylation at pH 4.8, even at 800 mM substrate. The loss of measurable activity upon replacement of the secondary butyl group of Ile with the methyl moiety of Ala shows that Ile-59 is crucial to normal HDC activity. Unfortunately, since no kinetic analysis could be made, it was not possible to dissect this effect into catalytic or substrate binding components or to assess the effect of the mutation on cooperativity.

Because the I59A mutation had such a strong effect on the enzyme, a more conservative mutation was made in which Ile-59 was converted to Val (I59V). Again, the activation of proenzyme proceeded at near-normal rates, and indeed is slightly faster than wild type (Table II). The processed enzyme was quite active and showed hyperbolic kinetics at pH 4.8 (Figure 5). As shown in Table II, K_m decreased slightly compared to wild type, although this observed difference is probably close to the level of experimental error. The observed k_{cat} is decreased about 3.5 times; this half-log difference is significant and reproducible. The specificity constant, k_{cat}/K_m, reflects the second-order rate constant for the reaction of free enzyme and substrate (Fersht, 1985); it is 3-fold lower for I59V than for wild type.

The I59V protein exhibited sigmoidal kinetics at pH 7.6 (Figure 6). The Hill number is 2.8 compared to 1.9 for wild type, showing that cooperativity is modestly increased by the mutation. The $S_{0.5}$ for the I59V mutant is 3 mM, some 7-fold lower than for wild type. V_m, and therefore k_{cat}, is decreased 9-fold compared to wild type. Analysis of a Hill plot for I59V (Figure 4) shows that the apparent K_m for the mutant T state is 8 mM, some 6-fold lower than for wild type. The K_m for the R state of I59V is 1 mM, 5-fold lower than for wild type. The increased Hill number and the shape of the profile indicate that the shift from T to R is slightly more abrupt for the mutant than for wild type.

One possible interpretation of the kinetic data for I59V, which provides a simple rationalization for the observed kinetic perturbations, is as follows. The active site for wild-type HDC includes a substrate binding pocket which is relatively snug stereochemically and electrostatically. That is, substrate

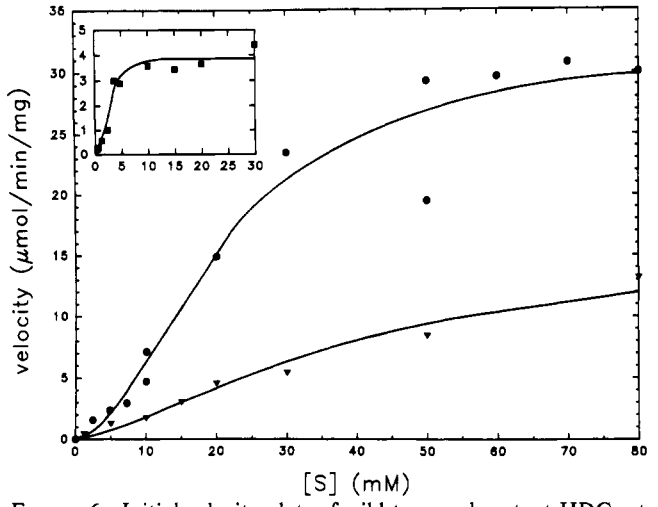

FIGURE 6: Initial velocity plots of wild-type and mutant HDCs at pH 7.6. The solid circles are wild type, and the downward triangles are Y62F. I59V is shown as solid squares in the inset figure, which has the same units on its axes as the main graph.

binding includes close van der Waals interactions with residues like Ile-59 and electrostatic interactions between the substrate imidazolium and residues like Asp-63, to be discussed in more detail below. The snug fit presumably aligns the substrate to optimize catalytic activity and maximize k_{cat}. When Ile-59 is converted to Val, in I59V, the binding pocket is enlarged, and the Michaelis or ES complex can assume a number of related conformations. This less restrictive binding is manifest as a decrease in K_m, as observed. At the same time, this less restrictive binding diminishes k_{cat}, as observed, because the substrate is not always optimally aligned. The effect of the mutation is more pronounced at pH 7.6 than at pH 4.8. This is probably because at pH 4.8 electrostatic effects between the positively charged substrate and Asp-63 are strong, helping to align the substrate. The strength of the ion pairing diminishes as the pH rises and His is deprotonated. With the electrostatic constraint reduced, the decrease in stereochemical selectivity is more noticeable.

Tyr-62 to Phe (Y62F). Because of its active-site proximity, Tyr-62 has figured in mechanistic speculations since the HDC crystal structure was first solved (Parks et al., 1985). It was, for example, suggested that Tyr-62 might function as a proton donor in autoactivation (Hackert et al., 1987). Later studies showed that the hydroxyl of Tyr-62 undergoes the largest movement in HDC upon substrate binding, and is involved in a network of hydrogen bonds as a result (Gallager et al., 1989). To quantify its importance, the hydroxyl group was removed by conversion to phenylalanine.

The half-time of autoactivation for Y62F is 17 h (Table II); this 3-fold increase over wild type is relatively small. It seems unlikely that Tyr-62 plays any specific and significant role, such as proton donation, in the activation process.

Once activated, Y62F shows hyperbolic kinetics at pH 4.8 (Figure 5), suggesting it is in the proton-induced R state. The K_m is 2.8 mM, 7-fold higher than that for wild type, while the k_{cat} is 90% of wild type, which is probably identical within the experimental error of the measurements.

At pH 7.6, Y62F is cooperative, with a Hill number similar to wild type. This suggests that the Tyr-62 hydroxyl does not play a major role in control of cooperative behavior or in HDC action. V_m, and therefore k_{cat}, is reduced about 2-fold, and $S_{0.5}$ increases by a factor of 2. The apparent affinity for substrate is reduced at both pH values, suggesting that the network of hydrogen bonds in which this residue participates (Figure 1) does help determine substrate binding, but is not as major a contributor as was once speculated. The effects on k_{cat} are less pronounced.

Asp-63 to Asn (D63N). As shown in Figure 1, Asp-63 forms a hydrogen bond or ion pair with the imidazolium of the histidine substrate. It was anticipated that this residue should have a strong effect on substrate binding and that alterations here would affect K_m. It was also possible that it might be involved, like Glu-66, in the pH-dependent cooperative activity of HDC. To test these putative roles, Asp-63 was converted to the amide, Asn.

The conversion of Asp-63 to Asn had no significant effect on the rate of autoactivation (Table II) which implies that Asp-63 is not involved in the serinolysis reaction leading to proenzyme cleavage and cofactor production. The k_{cat} for the mutant at pH 4.8 is the same, within experimental error, as that for wild-type HDC. This indicates that the residue is not involved in the catalysis of decarboxylation. In other words, residue 63 interacts with the Michaelis complex in essentially the same way as with the transition state. The alteration to Asn destabilizes the Michaelis complex and the transition state by the same amount.

At pH 4.8, D63N exhibits hyperbolic kinetics (Figure 5), and is presumably in the R state, but the K_m is 97 mM. This is the highest value we have been able to measure for any HDC mutant, 240 times higher than the K_m for wild type, and shows how crucial Asp-63 is for substrate recognition (again adopting the simplest interpretation of kinetic parameters). This observation suggests that the Asp-63 carboxylate is better than the neutral amide at stabilizing substrate binding. Asn has essentially the same stereochemistry as Asp and can, in principle, receive a hydrogen bond from the His ring. Apparently such a bond is not as strong as the wild-type interaction, which is probably a true ion pair. If all of the 240-fold difference observed in binding is ascribed to the difference in strength between the hydrogen bond and an ion pair, that differential interaction would be 3.2 kcal/mol. In fact, the poor binding of substrate to D63N could be partly due to differences in the electrostatic interactions and partly to a generalized distortion of the binding site.

As shown in Table II, the D63N protein has no measurable activity at pH 7.6. This is to be expected given the above results. We have seen that the K_m for the R state, at pH 4.8, of the D63N protein is 97 mM (Table II). Figure 4 suggests that T-state HDC, at pH 7.6, binds substrate 5–10-fold less well than the R state. Histidine substrate can only be made about 250 mM in the assay buffer due to reduced solubility at pH 7.6, and it is therefore experimentally difficult to obtain meaningful enzyme activity at this pH.

The differences in substrate binding between wild type and D63N and the differences between wild-type R states at pHs 4.8 and 7.6 were compared to assess the strength of the Asp-63–imidazolium interaction. In the latter case, the two R forms differ slightly in tertiary structure because the low-pH version has apparently bound at least one additional proton, although not necessarily at the substrate binding site. For this discussion, it is not unreasonable to assume that the two R forms are equivalent in structure except for the ionization state of acids and bases.

As discussed above, the loss of the wild-type ion pair interactions by D63N, in the pH 4.8 R state, is worth up to 3.2 kcal/mol. We can also examine wild-type R-state substrate binding at pHs 4.8 and 7.6. The wild-type K_m values are 0.4 mM (Table II) and 5 mM (Figure 4), respectively. This 12-fold difference corresponds to a loss of binding strength of

about 1.5 kcal/mol at pH 7.6 compared to pH 4.8. Why do we not see a larger difference, nearer to 3.2 kcal/mol? Presumably, histidine binds more poorly to the R state at pH 7.6 because the ion pairing between imidazolium and Asp-63 is diminished; as pH increases, the His side chain is deprotonated. The pK_a for His is likely to be higher in the carboxylate-rich active site of HDC than the 6.4 value commonly assumed for it. As a consequence, at pH 7.6 His is probably still partially protonated, and might even be roughly half-charged. We cannot accurately measure kinetic parameters at pH values much above 7.6, but presumably if the pH could be taken higher, and His completely deprotonated, the ion pair would be seen to account for even more than 1.5 kcal/mol. Integrating this notion with the mutation data for D63N, it seems safe to conclude that the pairing of the Asp-63 carboxylate with the substrate imidazolium is important to HDC action and that this interaction is stronger than simple hydrogen bonding by at least 1.5 kcal/mol and may be worth as much as 3.2 kcal/mol.

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