Site-Directed Alteration of the Active-Site Residues of Histidine Decarboxylase from *Clostridium perfringens*†

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ABSTRACT: To clarify the mechanism of biogenesis and catalysis by the pyruvoyl-dependent histidine decarboxylase (HisDCase) from *Clostridium perfringens*, 12 mutant genes encoding amino acid substitutions at the active site of this enzyme were constructed and expressed in *Escherichia coli*. The resulting mutant proteins were purified to homogeneity, characterized, and subjected to kinetic analysis. The results (a) exclude all polar amino acid residues in the active site except Glu-214 as donor of the proton that replaces the carboxyl group of histidine during decarboxylation and, since E214I and E214H are nearly inactive, indicate that Glu-214 is the essential proton donor; (b) demonstrate the importance to substrate binding of hydrophobic interactions between Phe-98, Ile-74, and the imidazole ring of histidine, and of hydrogen bonding between Asp-78 and N2 of the substrate; and (c) demonstrate a significant unidentified role for Glu-81 in the maintenance of the active-site structure. The proposed roles of these amino acid residues are consistent with those assigned on the basis of crystallographic evidence to the corresponding residues at the active site of the related HisDCase from *Lactobacillus* 30a [Gallagher, T., Snell, E. E., & Hackert, M. L. (1989) *J. Biol. Chem. 264*, 12737–12743]. Of the residues altered, only Ser-97 was *essential* for the autocatalytic serinolysis reaction by which this HisDCase, $(\alpha\beta)_6$, is derived from its inactive, pyruvate-free precursor, proHisDCase, π_6. The conservative substitution of Thr for Ser-97 yielded a mutant proHisDCase that cleaved very slowly ($t_{0.5}$ = 4 days) to yield a very weakly active HisDCase with an α-ketobutyroyl group replacing the pyruvoyl group at the active site. Replacement of Glu-214 by Ile or His slowed activation perceptibly; replacement of other polar groups near the active site did not influence that rate markedly. Proenzyme activation thus seems to be dependent mainly upon the uniquely reactive serine residue Ser-97 and does not involve the obvious participation of other residues as proton donors/abstractors.

The histidine decarboxylases (HisDCases)[1] from *Lactobacillus 30a* and from *Clostridium perfringens* are representative of a small but widely distributed group of enzymes that contain pyruvate as a covalently bound, catalytically essential prosthetic group [for reviews, see Recsei and Snell (1984) and van Poelje and Snell (1990a)]. The genes for these two HisDCases have been cloned and sequenced; each gene encodes a single peptide chain (the π chain) of a catalytically inactive proenzyme, proHisDCase (π_6). Although the π chains of the two proHisDCases are only 47% homologous (van Poelje & Snell, 1990b), much higher homology exists near their activation sites (Huynh & Snell, 1984; van Poelje & Snell, 1990b). Near pH 7, both proenzymes undergo an autocatalytic activation to yield the two peptide chains characteristic of the active enzymes, $(\alpha\beta)_6$. Activation occurs by nonhydrolytic serinolysis, during which the essential pyruvate residue at the amino terminus of the α subunit is generated from an internal serine residue of the proenzyme (Recsei & Snell, 1984; van Poelje & Snell, 1990a; Vanderslice et al., 1988).

The three-dimensional structure of HisDCase from *Lactobacillus* 30a is known and has permitted certain mechanistic features of the decarboxylation reaction to be defined (Gallagher et al., 1989). As shown in Figure 1, *all* of the amino acid residues implicated by these studies and by site-specific mutagenesis (McElroy & Robertus, 1989) in substrate binding and catalysis by the HisDCase from *Lactobacillus 30a* are conserved at the same relative positions in the HisDCase from *C. perfringens*.[2] In addition to the residues of Figure 1, the predominantly hydrophobic substrate binding pocket is lined with apolar groups, including the side chains of Ala-*153*, Phe-*195*, Ala-*80*, and Ile-*180* and the methyl groups of Thr-*258* and Prv: these residues, too, are conserved at the same relative positions in the clostridial enzyme. These observations strongly favor the view that activation of these two proenzymes and catalysis of decarboxylation by the resulting two HisDCases follow closely similar mechanisms in the two organisms. To test this conclusion and to delineate further the role of specific amino acid residues in activation and catalysis, we describe here the use of site-specific mutagenesis to obtain HisDCases from *C. perfringens* altered at each of the positions shown in Figure 1, together with the effects of such modification on activation, heat stability, and kinetic parameters of the resulting proteins.

Materials and Methods

Materials. Suppliers of materials were as follow: L-[carboxyl-^{14}C]histidine (56 mCi/mmol), [^{35}S]dATP (1350 Ci/mmol), and most enzymes and other cloning materials, New

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[1] Abbreviations: HisDCase, histidine decarboxylase; proHisDCase, prohistidine decarboxylase; IPTG, isopropyl β-D-thiogalactopyranoside; HisOMe, histidine methyl ester; kb, kilobase pair(s); SDS, sodium dodecyl sulfate; Prv, pyruvoyl group.

[2] Throughout this paper, amino acid residues are numbered in sequence from the amino terminus of the *proenzyme* (not including the encoded amino-terminal methionine residue that is removed during processing). Residue numbers in *italics* refer to proHisDCase from *Lactobacillus* 30a,while those in roman typeface refer to proHisDCase from *Clostridium perfringens*.

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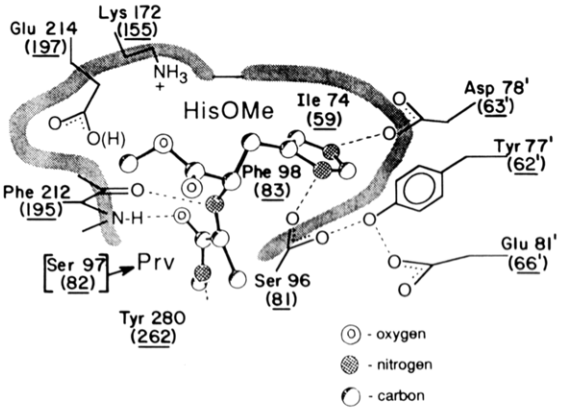

FIGURE 1: Schematic diagram [modified from Gallagher et al. (1989)] showing those amino acid residues of HisDCase from *Lactobacillus 30a* (underlined numbers) involved in key interactions with histidine methyl ester (HisOMe). The corresponding conserved amino acid residues of the HisDCase of *C. perfringens* are shown without underlining. The interactions shown are those demonstrated by X-ray crystallography for the HisDCase from *Lactobacillus* 30a (Gallagher et al., 1989) and inferred for the clostridial enzyme. Numbering of residues is based on the sequence of the π chains of the two proHisDCases; primed numbers refer to residues of an adjacent peptide chain.

England Nuclear or Promega Corp.; Sequenase DNA sequencing kit, U.S. Biochemicals; Sephacryl S-200 and Sephadex G-50, Pharmacia; DEAE-cellulose D-52, Whatman. The oligonucleotide primers used for sequencing and site-directed mutagenesis were synthesized locally on an Applied Biosystems Model 381A DNA synthesizer and purified by passage through Sephadex G-50 prior to use. Other chemicals were of reagent grade from standard sources.

The bacterial strains, their genotypes, and the growth conditions are described fully elsewhere (van Poelje & Snell, 1990b).

DNA Sequencing. Single-stranded DNA templates were sequenced in M13mp18 (Yannisch-Perron et al., 1985) by using a Sequenase DNA sequencing kit according to the instructions of the manufacturer. Use was made of 4 sequencing primers designed to anneal to the *hdc* gene at approximately 250-base intervals. Acrylamide gels [4–8%; acrylamide:bis(acrylamide) ratio, 19:1] were run on a Hoefer poker face sequencer.

Construction and Expression of Mutant hdc Genes. All DNA manipulations were performed using standard procedures (Maniatis et al., 1983). The *Eco*RI–*Pst*I fragment (1.1 kb) of expression vector pVP-4 (van Poelje & Snell, 1990b), which contains the complete clostridial *hdc* gene, was subcloned into M13mp18. *Escherichia coli* BW313 (dut^-ung^-) was transfected with the recombinant phage, and uracil-containing single-stranded phage DNA was purified. Twelve site-specific mutant *hdc* genes were subsequently constructed by the method of Kunkel et al. (1987) with use of the oligonucleotide primers (20–26 bases) described in Table I. The mutant strands of the resulting heteroduplex molecules were selected by transfection into *E. coli* JM103Y (dut^+ung^+). Single-stranded DNA was isolated from the resultant phage and screened for the desired mutation(s) by single- or double-lane DNA sequencing. One template was selected for each mutant and the base change(s) verified by sequencing the appropriate region. Replicative-form DNA corresponding to these templates was isolated, and the mutant *hdc* genes were excised by digestion with *Eco*RI and *Pst*I. The size of each mutant gene was verified as correct by agarose gel electrophoresis using the wild-type gene as one of the molecular weight standards. The mutant genes were subsequently cloned into appropriately restricted pUC8 and expressed in *E. coli* GM1 under control of the *lac* promoter as described for the wild-type gene (van Poelje & Snell, 1990b). Cultures were grown for 12–18 h at 37 °C in double-strength TYE medium (2% tryptone, 1% yeast extract, and 0.5% NaCl, pH 7.0) supplemented with 200 μg/mL carbenicillin and 0.5 mM IPTG for enzyme induction.

Table I: Codon Changes in the *hdc* Gene of *C. perfringens* Used To Generate Mutant HisDCases

codon change	position of mutagenic primer in *hdc* gene[a]	amino acid change in mutant HisDCase[b]
ATA to *GA*A	252–273	I74(*59*)E
TAT to T*T*T	258–280	Y77(*62*)F
GAT to G*C*T	258–282	D78(*63*)A
GAA to *C*AA	273–295	E81(*66*)Q
TCA to *A*CA	317–342	S96(*81*)T
TCA to *A*CA	317–342	S97(*82*)T
TTC to *GC*C	322–342	F98(*83*)A
AAG to A*TT*	547–568	K172(*155*)I
GAA to GA*T*	666–691	E214(*197*)D
GAA to *CA*C	666–691	E214(*197*)H
GAA to *AT*A	666–691	E214(*197*)I
TAT to T*T*T	871–892	Y280(*262*)F

[a] The wild-type *hdc* gene sequence and the corresponding amino acid sequence have appeared elsewhere (van Poelje & Snell, 1990b). [b] The notation I74(*59*)E indicates that Ile-74 in proHisDCase from *C. perfringens* corresponds to Ile-*59* in proHisDCase from *Lactobacillus* 30a and has been changed to Glu in this mutant enzyme. Subsequent entries use the same notation.

Purification of Mutant HisDCases. Each mutant HisDCase was purified to homogeneity from 1- or 2-L cultures as described earlier for the wild-type enzyme (van Poelje & Snell, 1990b). Prior to the heat step in the purification procedure, the temperature stability of each mutant protein was determined by incubating 50-μL portions of the redissolved 45–90% ammonium sulfate fraction at a range of temperatures (60–95 °C) for 4 min. Each sample was cooled on ice and centrifuged, and the enzyme activity was determined in the supernatant solution. Purified proteins were stored in 0.2 M ammonium acetate, pH 4.5, at 4 °C.

HisDCase and Protein Assay. Enzyme activity was measured by the release of $^{14}CO_2$ from carboxyl-labeled L-histidine (Rescei & Snell, 1970; Ichiyama, 1970) in 0.2 M ammonium acetate/0.4 M magnesium sulfate, at pH values that varied from 3.0 to 6.0. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Sodium Dodecyl Sulfate Gel Electrophoresis. Purity of the mutant HisDCases was confirmed by electrophoresis of the denatured protein in the presence of 1% SDS on polyacrylamide slab gels (Laemmli, 1970). Protein bands were visualized with Coomassie blue (Fairbanks et al., 1979).

Results

Mutagenesis. Twelve *hdc* genes containing the codon changes shown in Table I were constructed as described under Materials and Methods and successfully expressed in *E. coli* GM1. The probable arrangement and roles of the targeted amino acid residues at the active site of HisDCase (or the activation site of proHisDCase) are shown in Figure 1. The amino acid residues altered fall into three categories: (a) those involved directly or indirectly in binding of histidine at the active site (Asp-78, Ser-96, Ile-74, Phe-98, Tyr-77, and Glu-81); (b) those that may serve as potential proton donors in the decarboxylation of L-histidine (Glu-214, Lys-172, and Tyr-77); and (c) those that provide polar groups in the active site that might facilitate conversion of proHisDCase to active HisDCase

Table II: Catalytic Properties of Homogeneous HisDCases Obtained by Expressing the Wild-Type and Mutant Genes of *C. perfringens* in *E. coli*

mutation[a,b]	temp stability (°C)[c]	pH optimum[d]	K_m (mM)[e]	V_{max} [μmol/(min·mg)]	K_{cat}/K_m (min^{-1} mM^{-1})
wild type	80–85	4.5	0.2	25.0	5208
I74(*54*)E	75–80	4.75	1.5	12.5	347
Y77(*62*)F	70–75	4.5	0.35	18.2	2166
D78(*63*)A	80–85	4.25	3.1	0.33	4.4
E81(*66*)Q	75–80	4.5	6.6	14.7	37
S96(*81*)T	85–90	4.5	0.9	28.6	1324
S97(*82*)T	80–85	ND[h]	0.9	(0.016)[g]	(0.74)
S97(*82*)A[f]	60–65	*i*	*i*	*i*	*i*
F98(*83*)A	80–85	4.5	4.7	1.4	12.4
K172(*155*)I	80–85	5.0	1.35	12.4	383
E214(*197*)D	80–85	4.5	0.06	1.0	694
E214(*197*)H	75–80	4.0	0.043	0.087	84.3
E214(*197*)I	80–85	3.5	0.008	0.03	166
Y280(*262*)F	85–90	4.5	4.16	26.6	266

[a] The notation of mutant enzymes is given in Table I. [b] Mutant enzymes E214H and E214I, which were partially present in proenzyme form (Figure 2) after a 2-h activation time, were fully activated by incubation in 1.0 M ammonium phosphate, pH 8.0 (12 h, 37 °C) for 12 h. Mutant S97T was incubated for 10 days (cf. Figure 3) prior to assay. [c] Temperature stabilities were determined as described under Materials and Methods. The enzymes were stable (for 4 min) at the lower temperature of the range shown; loss of activity (or denaturation with S97A and S97T) was first observed at the higher temperature of the range. [d] This value was determined by using substrate concentrations at least 20 times the K_m value at pH 4.5. [e] K_m values were determined at pH 4.5, the pH optimum of wild-type HisDCase. [f] This mutant protein, described earlier (van Poelje & Snell, 1990b), is included here for comparative purposes. [g] This assay was linear for only 20 min; this figure is probably a minimum value. [h] Not determined. [i] ProHisDCase only; activation does not occur.

(Ser-97, Glu-214, Lys-172, Asp-78, Tyr-77, Glu-81, and Tyr-280). In general, the active-site/activation-site residues were replaced by residues that could, in principle, perform the same role as that postulated for the original residue (e.g., E214D) or would clearly be unable to perform that role (e.g., E214I).

Purification and Characterization of Mutant HisDCases. All of the mutant HisDCases were purified to apparent homogeneity; yields of 50–90 mg of mutant enzyme per liter of culture medium were routinely achieved. These yields are considerably higher than those reported earlier for the cloned wild-type enzyme (van Poelje & Snell, 1990b). They result from use of double-strength TYE medium instead of single-strength medium employed initially; cell growth was 1.7-fold higher in the richer medium.

All 12 mutant enzymes eluted from the Sephacryl S-200 column (used in the last step of the purification procedure) in the same volume as the cloned wild-type enzyme, indicating that each mutant enzyme has the same molecular weight value, corresponding to an $(\alpha\beta)_6$ structure. Upon gel electrophoresis under denaturing conditions, all but one (S97T) of the mutant enzymes gave two bands identical in R_f values with the α (M_r 24 887) and β (M_r 10 526) subunits of the cloned wild-type enzyme. After a 2-h activation period, S97T gave only a single band (M_r 36 000) corresponding to the π subunit of pro-HisDCase, while preparations of E214I and E214H showed three bands, corresponding to α, β, and π subunits (Figure 2A), indicating that these proenzymes were not completely activated within this time. Upon longer incubation, however, all three of these mutant enzymes activated completely; activation of S97T ($t_{1/2}$ = 4 days, Figure 2B) was by far the slowest. The β subunit of the I74E mutant migrated more slowly than that of the wild-type enzyme, possibly because its additional negative charge decreases SDS binding. Similar effects of charge on migration in SDS gels have been observed elsewhere (Rescsei & Snell, 1981a).

Comparative Properties of the Mutant HisDCases. The consequences of altering amino acid residues in or near the histidine binding site of HisDCase (Figure 1), as reflected by the pH optimum, heat stability, and catalytic parameters of the enzymes, are summarized in Table II. Each alteration diminished the catalytic efficiency (K_{cat}/K_m) of the enzyme.

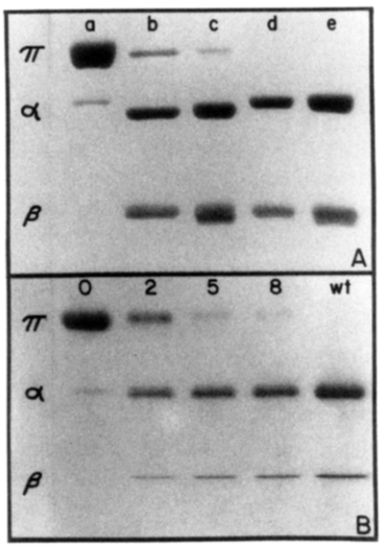

FIGURE 2: Electrophoretic characterization (SDS–PAGE) of purified preparations of mutant and wild-type (pro)HisDCases under denaturing conditions. In panel A, proteins are (a) S97T, (b) E214I, (c) E214H, (d) E214D, and (e) wild type. In panel B, the time of activation of the S97T mutant enzyme is given (in days) at the head of each column. The approximate molecular weights of the proenzyme π chains and the α and β chains of the active enzyme are 35 000, 25 000, and 10 500, respectively (van Poelje & Snell, 1990b). See text for further details.

The greatest reduction was evident in D78A; other notable reductions occurred in the order S97T > F98A > E81Q > E214H > E214I > Y280F. These mutant enzymes were only 5% or less as efficient as wild type. This measurement, however, is misleading: based upon V_{max}, S97T, E214I, E214H, D78A, and E214D are the least effective catalysts with activities ranging from about 0.06% to 4% those of the wild-type enzyme. As discussed below, most of the observed effects are consistent with the roles assigned to the corresponding residues at the active site of the *Lactobacillus* 30a HisDCase (Gallagher et al., 1989).

In the imidazole pocket, Asp-78 stands out as one of the key residues involved in the action of this enzyme. Its replacement by Ala resulted in a 15.5-fold increase in K_m and a 76-fold decrease in V_{max}. These results emphasize the im-

portance of the hydrogen bond formed between this residue and N2 of the substrate (Figure 1) in binding and positioning the substrate for catalysis. Ser-96 also hydrogen bonds to the imidazole group (N1) of the substrate. A similar test of the contribution of this bond to activity could not be made, since activation of proHisDCase requires liberation of a carboxyl group at this position that can, in principle, form this same hydrogen bond from any amino acid residue. Replacement of Ser-96 by Thr (S96T, Table II), in fact, increases K_m only slightly and does not change V_{max} appreciably (Table II). Phe-*83* and Ile-*59* both are involved in hydrophobic interactions with the substrate imidazole in the *Lactobacillus* enzyme (Figure 1). The corresponding residues in the clostridial enzyme (Phe-98 and Ile-74) also contribute greatly to binding and orientation of substrate (Table II) with Phe-98 contributing somewhat more than Ile-74.

The replacement of Tyr-77 by Phe has only a minor effect on the catalytic efficiency of the clostridial enzyme. In the HisOMe–HisDCase complex of the *Lactobacillus* enzyme (but not in the free enzyme), the corresponding residue (Tyr-*62*) hydrogen bonds with carboxyl groups of both Ser-*81* and Glu-*66* (Figure 1); the resulting network was thought to stabilize the enzyme–substrate complex. The importance of this network for catalysis is discounted by the results of Table II; they also eliminate the remote possibility (Gallagher et al., 1989) that this Tyr residue functions as a proton donor during decarboxylation. However, replacement of Glu-81 by Gln resulted in a 33-fold increase in K_m without greatly affecting V_{max}; the result affirms a significant but undefined role for Glu-81 in substrate binding. Modeling studies (M. L. Hackert, private communication) suggest that the effect of Glu-81 may be mostly electrostatic: in the absence of substrate, it helps impart a diffuse negative charge to the imidazole binding pocket that assists in binding and is substantially neutralized when substrate is bound.

Glu-214 and Lys-172 are the only residues with ionizable side chains close to the carboxyl group of the bound substrate (Figure 1). Glu-214 appears to be essential to catalysis; its replacement by Asp results in a 25-fold decrease in V_{max}, while its replacement by His or Ile nearly abolishes activity, as evidenced by decreases in V_{max} of 287- and 833-fold, respectively (Table II). Both E214H and E214I have much higher affinities for histidine (as suggested by decreased K_m values) than the wild-type enzyme. The high activity of the K172I, Y77F, and Y280F enzymes (Table II) rules out Lys-172, Tyr-77, and Tyr-280 as catalytic residues.

Effects of Amino Acid Replacements on the pH Optimum for Enzymic Activity. Most of the mutational changes in the clostridial HisDCase resulted in relatively minor changes in the pH dependency of decarboxylation (Table II). In E214I, however, the pH optimum was reduced by a full pH unit (Figure 3); the pH at which the lowest K_m was observed also was reduced. Glu-214 of the clostridial enzyme corresponds to Glu-*197* of the *Lactobacillus* enzyme; this residue probably serves as the proton donor during the decarboxylation reaction (Gallagher et al., 1989). A logical explanation for the shift in pH optimum of E214I might be that, within the limits permitted by protein stability, the absence of Glu as a proton donor is partially compensated for by the increased proton concentration. This effect could be mediated by partial suppression of ionization of the carboxyl group (pK_a = 1.8) of histidine, which should facilitate its entry into the hydrophobic substrate binding pocket [thus explaining as well the differential effect of pH on the K_m value for histidine in this mutant enzyme (Figure 3)]. Water or the proton thus cotransported into the hydrophobic pocket could then supply the proton required to replace the carboxyl group.

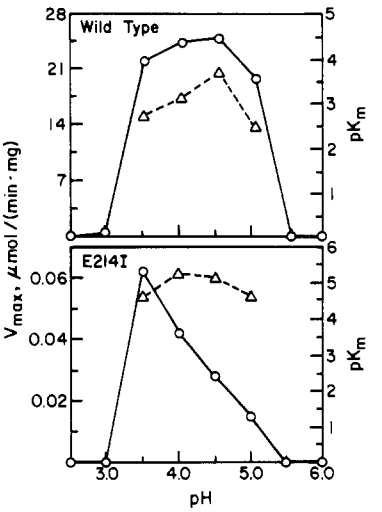

FIGURE 3: Effect of pH on the V_{max} (O) and K_m (Δ) of (top) wild-type clostridial HisDCase and (bottom) its E214I mutant.

ProHisDCase Activation. By converting each of the polar residues near the Ser96–Ser97 bond of proHisDCase (Figure 1) to nonpolar residues, we hoped to locate one or more of the proton donor/abstractor residues thought to be necessary for activation. However, of the clostridial mutants listed in Table II, only S97A failed to activate. S97T showed barely detectable activation after 2 h but was activated completely after 10 days (Figure 2B). The traces of residual proenzymes observed on SDS gel electrophoresis of E214I and E214H (Figure 2A) show that these proteins also activate somewhat more slowly than the remaining mutant enzymes of Table II. These proenzyme traces were not observed in samples incubated for 12 h at 37 °C in 1.0 M ammonium or potassium phosphate, pH 6.0–9.0, conditions similar to those used for optimal activation of proHisDCase from *Lactobacillus* 30a (Recsei & Snell, 1981b). Although replacement of Glu-214 thus affects the rate of activation, the residue is not an essential participant in this process.

DISCUSSION

The amino acid residues to be replaced in clostridial HisDCase were selected on the assumption that their arrangement and orientation at the active site of this enzyme would be similar to those of the corresponding residues in HisDCase from *Lactobacillus* 30a, for which a refined (2.5 Å) three-dimensional structure is known. The validity of this assumption was confirmed by the results of this study: not only were the properties of the mutant clostridial HisDCases consistent with the mechanism of decarboxylation proposed for the *Lactobacillus* enzyme (Gallagher et al., 1989), but also in those cases where corresponding mutants of the *Lactobacillus* enzyme have been studied (McElroy & Robertus, 1989), a pronounced similarity in the catalytic consequences of the mutations was observed. For example, in both the *Lactobacillus* and clostridial HisDCases, replacement of the key Glu-214(*197*) residue by Asp gave enzymes with reduced activity, while replacement by a nonpolar residue resulted in a dramatic reduction in both K_m and V_{max}. Similarly, replacement of Glu-81(*66*) by Gln resulted principally in a substantial increase in K_m in both cases.

The ionization state of Glu-214(*197*) in these enzymes is unknown: if this residue is mostly ionized, it could provide an "electrostatic push" favoring elimination of the negatively charged substrate carboxyl group of bound histidine as CO_2.

Alternatively, by cycling between its unionized and ionized forms, it can provide the proton required to replace the leaving carboxyl group (McElroy & Robertus, 1989; Gallagher et al., 1989). Both factors probably play a role. Electrostatic repulsion between an ionized Glu-214(*197*) and the incoming charged carboxylate group of histidine probably explains in part the reduced affinity of HisDCase for histidine as compared to HisOMe, a difference to which the hydrophobic nature of the carboxyl pocket also contributes greatly (Alston & Abeles, 1987). The greatly decreased K_m value for histidine exhibited by mutants such as E214I, where such repulsion is lacking while hydrophobicity is actually increased, also points to the importance of such charge effects. However, a primary role for Glu-214 as proton donor is favored by these results. Thus, the systematic replacement of other possible proton donors near the α-carbon atom of bound substrate in clostridial HisDCase (Lys-172, Tyr-280, Tyr-77, and Glu-81; Figure 1) by nonpolar amino acid residues has eliminated them as potential donors, leaving only Glu-214 to play this role. Asp substitutes inefficiently for Glu-214, but replacement by Ile nearly abolishes catalytic activity (Table II) and provides convincing evidence for the important catalytic role of this residue. The pH dependency for activity of wild-type enzyme (Figure 3) shows an optimum at pH 4.5, consistent with presence of a proton donor with a low pK_a value similar to that of Glu (pK_a = 4.25). The reduced pH optimum of the E214I mutant (Figure 3) supports this role as well: in the absence of the normal proton donor (Glu-214), higher proton concentrations increase the extremely low rate of decarboxylation observed. E214H shows a more modest but highly significant decrease [0.5 pH unit (Table II)] in its pH optimum and also shows a decreased K_m value for histidine. This mutant was constructed to test the proposition that a positively charged residue replacing Glu-214 might facilitate entry of the negatively charged substrate carboxyl group into the hydrophobic pocket of the active site and also provide a proton donor group to replace Glu-214. Both expectations are only partially met: although this protein has a lower K_m value than the wild-type enzyme, the value is higher than that for E214I, and although E214H is a far less efficient catalyst for decarboxylation than the wild-type enzyme, it is superior to E214I in this respect. Because of uncertainties concerning both conformational effects of the mutation and the pK_a of ionizable groups in such a hyrophobic environment, further interpretation of these effects is unjustified.

An unexpected observation was that substitution of Phe for Tyr-77 had little effect on catalytic efficiency. In the *Lactobacillus* enzyme, the corresponding residue is appropriately positioned to form a network of hydrogen bonds (Glu-*66* → Tyr-*62* → Ser-*81*) in the HisOMe–HisDCase complex that was viewed as stabilizing the active-site region and possibly providing a conduit for protons to access the active site (Gallagher et al., 1989). The three corresponding residues (Glu-81, Tyr-77, and Ser-96) are conserved in the clostridial enzyme, but the high activity of the mutant protein Y77F demonstrates that Tyr-77 plays no essential role in binding or catalysis. However, both this mutant enzyme and E81Q show significantly reduced stability to heat (Table II) that indicates conformationally important stabilizing roles for these residues. The conservative substitution of Thr for Ser in S96T has little effect on either heat stability or catalytic activity, but since Thr can obviously fill the roles ascribed to Ser in this case, the result is not mechanistically informative.

Despite the similarity in biogenesis and catalytic mechanisms revealed by these studies, significant differences in kinetic properties exist between the clostridial and *Lactobacillus* HisDCases which must reflect conformational differences near their active sites. For example, the clostridial enzyme has a lower turnover number than the *Lactobacillus* enzyme (1042 vs 2800) and unlike the latter enzyme requires a high ionic strength (ca. 1.5 M) for maximal activity (Recsei & Snell, 1983; van Poelje & Snell, 1990b). In addition, the clostridial enzyme has a narrower pH range for activity (pH 3.0–5.0, Figure 3) than the *Lactobacillus* enzyme (pH 3.0–7.6, Recsei & Snell, 1970). The effective lack of activity above pH 5.5 suggests that in order to decarboxylate histidine in vivo the clostridial enzyme would require compartmentalization in an acidic microenvironment. It has not been ruled out that at physiological pH values this enzyme may have a function other than production of histamine.

Ser-97 is the only residue implicated by this and previous studies (van Poelje & Snell, 1990b) as *essential* for pro-HisDCase activation. these results suggest that serinolysis of the Ser-96–Ser-97 bond is mediated mainly by the uniquely reactive hydroxyl group of this serine residue, and proceeds without any obvious requirement for other residues as proton donors or abstractors. Ser-97(*82*) was already known to be essential for normal proenzyme activation since (a) it is the precursor of the essential pyruvoyl residue and (b) its replacement by an alanine residue [in the clostridial enzyme (van Poelje & Snell, 1990b)] or by a dehydroalanyl residue [in the *Lactobacillus* enzyme (van Poelje & Snell, 1988)] yields a proenzyme species that does not activate. Its replacement by Thr (Table II) yields a proenzyme that activates very slowly to yield a very weakly active enzyme with an α-ketobutyroyl group in place of a pyruvoyl group blocking the NH_2-terminus of the α chain.[3] The results are analogous to those reported previously (Vanderslice et al., 1988) for the corresponding *Lactobacillus* mutant, S*82*T. While the clostridial mutant (S97T) has the same temperature stability as the wild-type enzyme (Table II), the Ala mutant (S97A) denatures at a temperature some 20 °C lower than wild type. These findings strengthen the interpretation (van Poelje & Snell, 1990b) that decreased temperature stability of S97A results from lack of a hydrogen bond involving the hydroxyl group of Ser-97 in the wild-type proenzyme (or of Thr-97 in S97T).

Finally, as in all site-directed mutagenesis studies, it is difficult to determine whether changes in the catalytic parameters of the mutant enzymes are due to conformational changes induced by the amino acid substitution or whether they are an indication of the catalytic importance of the residue in question. Three observations suggest that the three-dimensional structures of the mutant enzymes studied here are closely similar to that of the wild-type enzyme: (a) with exceptions already noted, the temperature stability of these mutant enzymes is similar to that of the wild type enzyme (Table II); (b) although activation of proHisDCase can be considered an intramolecular enzymic reaction and is sensitive to conformational changes [e.g., it is prevented by concentrations of denaturing agents below those necessary for complete denaturation (Recsei & Snell, 1981b)], it proceeds rapidly and normally to yield active enzymes in most of these mutants; and (c) the subunit structure of the activated mutant enzymes is the same in all cases as that of wild-type enzyme. Nevertheless, assignment of specific roles to individual residues

[3] The presence of an α-ketoacyl group in activated S97T was confirmed by titration with phenylhydrazine (Riley & Snell, 1968; Vanderslice et al., 1988) and identified as the α-ketobutyroyl group by its reduction to α-aminobutyrate by $NaCNBH_3$ in the presence of NH_3 (Huynh et al., 1984) by the referenced procedures.

must remain tentative until the three-dimensional structures of these proteins have been determined.

Registry No. HisDCase, 9024-61-7; proHisDCase, 39346-25-3; Glu, 56-86-0; Asp, 56-84-8; Ile, 73-32-5; Phe, 63-91-2; Ser, 56-45-1.

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Fluorescent Oligopeptide Substrates for Kinetic Characterization of the Specificity of *Astacus* Protease†

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ABSTRACT: The design of fluorescent N-dansylated oligopeptides based on the tubulin cleavage pattern by *Astacus* protease yields substrates that are turned over up to 10^5 times faster than those presently available. On the basis of this study, an optimal substrate for *Astacus* protease contains seven or more amino acids and minimally requires at least five amino acids. Direct examination of the formation and breakdown of the ES complex shows its formation occurs within milliseconds at 25 °C. The best heptapeptide substrate, Dns-Pro-Lys-Arg-Ala-Pro-Trp-Val, is cleaved only between the Arg–Ala (P_1–P_1') bond with kinetic parameters k_{cat} = 380 s^{-1} and K_m = 3.7 × 10^{-4} M. The presence of Lys or Arg in the P_1 and P_2 positions yields high-turnover substrates. In the P_3 position, the enzyme prefers Pro > Val > Leu > Ala > Gly, following the same order of preference seen in the tubulin cleavage pattern. Substitution of Leu for Ala in P_1' and of Ser for Pro in P_2' decreases activity by 10^5- and 10^2-fold, respectively. In position P_3', substitution of Trp for Leu leaves the activity unaltered. However, introduction of the Trp fluorophore greatly enhances the sensitivity of the assay due to a 10-fold increase in indole fluorescence for cleavage of any peptide bond between the tryptophan and the dansyl group. Such an energy-transfer-based assay should have widespread use for detection of neutral proteases. The relationship of *Astacus* protease to a recently sequenced bone morphogenetic protein and to metalloproteinases which share the putative zinc binding sequence HExxHxxGxxH is discussed.

Astacus protease, an endopeptidase from the digestive tract of the freshwater crayfish *Astacus fluviatilis*, is a monomeric protein of 200 amino acids (Titani et al., 1987; Vogt et al., 1989). This invertebrate protease has been considered to represent a new family of proteolytic enzymes because of its unusual properties (Zwilling & Neurath, 1981). Specific, naturally occurring inhibitors of *Astacus* protease have not been detected, and catalytically essential amino acid residues have not been identified (Torff et al., 1980).

We recently demonstrated that *Astacus* protease contains 1 mol of catalytically essential zinc per mole of protein (Stöcker et al., 1988). Furthermore, despite the absence of overall

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