A Familiar Motif in a New Context: the Catalytic Mechanism of Hydroxyisourate Hydrolase†

Aniruddha Raychaudhuri and Peter A. Tipton*

Department of Biochemistry, University of Missouri, Columbia, Missouri 65211

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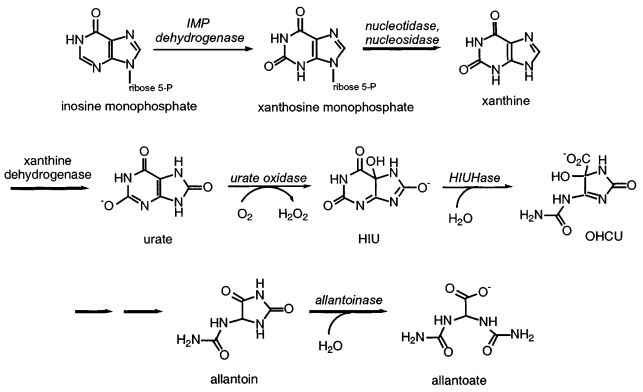
ABSTRACT: Hydroxyisourate hydrolase is a recently discovered enzyme that participates in the ureide pathway in soybeans. Its role is to catalyze the hydrolysis of 5-hydroxyisourate, the product of the urate oxidase reaction. There is extensive sequence homology between hydroxyisourate hydrolase and retaining glycosidases; in particular, the conserved active site glutamate residues found in retaining glycosidases are present in hydroxyisourate hydrolase as Glu 199 and Glu 408. However, experimental investigation of their roles, as well as the catalytic mechanism of the enzyme, have been precluded by the instability of 5-hydroxyisourate. Here, we report that diaminouracil serves as a slow, alternative substrate and can be used to investigate catalysis by hydroxyisourate hydrolase. The activity of the E199A protein was reduced 400-fold relative to wild-type, and no activity could be detected with the E408A mutant. Steady-state kinetic studies of the wild-type protein revealed that the pH-dependence of V_{max} and V/K describe bell-shaped curves, consistent with the hypothesis that catalysis requires two ionizable groups in opposite protonation states. Addition of 100 mM azide accelerated the reaction catalyzed by the wild-type enzyme 8-fold and the E199A mutant 20-fold but had no effect on the E408A mutant. These data suggest that Glu 408 acts as a nucleophile toward the substrate forming a covalent anhydride intermediate, and Glu 199 facilitates formation of the intermediate by serving as a general acid and then activates water for hydrolysis of the intermediate. Thus, the mechanism of hydroxyisourate hydrolase is strikingly similar to that of retaining glycosidases, even though it catalyzes hydrolysis of an amide bond.

In tropical legumes such as soybean, atmospheric nitrogen that is fixed as ammonia through the action of nitrogenase is rapidly synthesized into inosine monophosphate. Through a series of oxidative and hydrolytic reactions, IMP is converted to urate and then to allantoin and allantoate, which are collectively referred to as the ureides. The enzymes that accomplish these transformations constitute the ureide pathway (Scheme 1) (*1*). Fixed nitrogen appears in allantoin and allantoate within 10 min (*2*); the ureides move from the root of the plant into the xylem where they travel to the rest of the plant to deliver nitrogen that is used to support amino acid biosynthesis. Up to 95% of the nitrogen in the xylem is present as one of the ureides (*3*).

Urate oxidase is a component of the ureide pathway; indeed, it is widely reported that urate oxidase catalyzes the conversion of urate directly to allantoin. However, more detailed analysis has established that the true product of the urate oxidase reaction is 5-hydroxyisourate (*4*, *5*). HIU[1] has a half-life of about 30 min at neutral pH and 17 °C, and its decomposition yields allantoin quantitatively under many in vitro conditions. However, for a variety of reasons, the nonenzymatic production of allantoin from HIU is not likely to be physiologically relevant, and an enzyme that utilizes HIU as its substrate has been purified from soybean root nodules and characterized (*6*).

Scheme 1



The novel enzyme was shown to catalyze the hydrolysis of HIU and was therefore designated HIU hydrolase. Enzyme-catalyzed hydrolysis of HIU occurs via the addition of water across the N1−C6 bond and generates OHCU; the pathway for nonenzymatic conversion of HIU to allantoin begins with this reaction as well (*5*). The recent cloning and sequencing of the HIUHase gene (*7*) revealed extensive sequence homology with retaining glycosidases and suggested that it falls within Family 1, as defined by Henrissat

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* To whom correspondence should be addressed. Telephone: (573) 882-7968. Fax: (573) 884-4812. E-mail: tiptonp@missouri.edu.

[1] Abbreviations: Abg, *Agrobacterium* β-glucosidase; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHES, 3-(cyclohexylamino)-ethanesulfonic acid; DTT, dithiothreitol; HIU, 5-hydroxyisourate; MES, 2-(*N*-morpholine)ethanesulfonic acid; OHCU, 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline; PMSF, phenylmethanesulfonyl fluoride; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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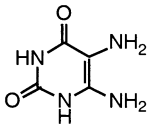
(see http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html (*8*)). In particular, two glutamate residues that are known to be at the active site in retaining glycosidases appear in HIUHase as Glu 199 and Glu 408. We found that mutation of either Glu to Ala resulted in catalytically inactive enzyme (*7*). However, the instability of HIU precluded more detailed characterization of the mutant enzymes and of the catalytic mechanism of the wild-type enzyme, as well. We now report that diaminouracil is a slow substrate for HIUHase; since it is more stable than HIU under the reaction conditions and does not need to be generated in situ, it is well-suited for mechanistic studies of HIUHase. With diaminouracil, we have been able to characterize the pH dependence of the kinetic parameters, the effect of alternative nucleophiles on the catalytic reaction, and quantitatively characterize the kinetic properties of the mutant enzymes. The results of these studies suggest that the HIUHase reaction proceeds through the formation of a covalent adduct between the substrate and the enzyme, in a mechanism that is strikingly reminiscent of that employed by retaining glycosidases.

MATERIALS AND METHODS

Purification of Recombinant HIUHase. Construction of the plasmid for expression of His-tagged HIUHase in *Escherichia coli* cells was reported previously (*7*). HIUHase is expressed well in this system, but most of the protein appears as inclusion bodies. Therefore, in the present work the protein in the inclusion bodies was purified and refolded. *E. coli* BL21(DE3)pLysS cells harboring the expression plasmid were grown at 30 °C in LB media to mid-log phase, and expression was induced by the addition of IPTG to 0.4 mM. The cells were harvested by centrifugation after 5 h. The cell paste from a 1 L culture was resuspended in 20 mL of 50 mM HEPES, pH 7.5, containing 0.5 M NaCl, 1 mM PMSF, 5 mM DTT, and 7 mg of lysozyme. The suspension was incubated for 1 h at 37 °C, and Triton X-100 was added to a final concentration of 1% (v/v). The sample was sonicated for 30 s intervals, followed by cooling on ice, until it was clarified. DNase I was added to a final concentration of 2 μg/mL, and the solution was incubated at 37 °C for 1 h. The inclusion bodies were sedimented by centrifugation at 31 000*g* for 45 min at 4 °C; the pellet was washed twice with TBS containing 1% (v/v) Triton X-100 and centrifuged as above. The pellet was solubilized in 2 mL of 50 mM HEPES, pH 7.5, containing 25 mM DTT and 6 M guanidinium HCl, and incubated at 4 °C for 1 h. The protein solution was placed in an ice bath, and solid ammonium sulfate was added slowly with stirring to 60% saturation. The solution was stirred for an additional 20 min and centrifuged at 31 000*g*. The supernatant was discarded, and the protein pellet was dissolved in a small volume of 50 mM potassium phosphate, pH 7.5, containing 0.5 M NaCl, and 4 M guanidinium HCl (buffer A) supplemented with 10 mM imidazole.

The soluble protein was applied to a 10 mL Fast Flow Chelating Sepharose column that was charged with Ni^{2+} and equilibrated in buffer A containing 10 mM imidazole. The column was washed with 20 mL of buffer A containing 100 mM imidazole, and HIUHase was eluted by washing with buffer A containing 140 mM imidazole. Fractions containing HIUHase were pooled and concentrated by ultrafiltration. Several rounds of dilution with 0.1 M Tris, pH 7.5, containing 4 M guanidinium HCl and 0.1 M NaCl, and reconcentration were conducted to exchange the buffer and concentrate HIUHase to 20 mg/mL.

Chart 1



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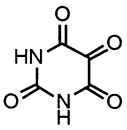

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Appropriate conditions for refolding HIUHase were identified using the Foldit Screen Kit (Hampton Research); samples of denatured HIUHase were diluted into the test buffers, incubated at 4 °C for 12 h, and then assayed for catalytic activity. The most effective buffer for refolding HIUHase contained 55 mM Tris, pH 8.2, 264 mM NaCl, 11 mM KCl, 2.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, and 0.44 M sucrose. In subsequent HIUHase preparations, concentrated, denatured HIUHase was diluted 20-fold into the refolding buffer at 4 °C and incubated for 12 h. The protein solution was then dialyzed against 50 mM potassium phosphate, pH 7.5 containing 50 mM NaCl and concentrated by ultrafiltration. The sample was then chromatographed on a Superdex-200 gel filtration column (1.2 × 100 cm) equilibrated in 50 mM potassium phosphate, pH 7.5 containing 50 mM NaCl, to separate the active protein from inactive protein aggregates. Fractions containing active HIUHase were pooled, concentrated, supplemented with glycerol to 10% (v/v), and stored at −80 °C.

HIUHase Assays. Diaminouracil, **1**, (5,6-diamino-2,4-dihydroxypyrimidine) and alloxan, **2**, (Chart 1) were obtained from Aldrich and tested as alternative substrates for HIUHase. Alloxan was not a substrate, but diaminouracil proved to be a slow substrate. The absorbance maximum of diaminouracil varied from 261 nm at low pH to 284 nm at high pH. The catalytic reaction was monitored spectrophotometrically by measuring the decrease in absorbance at the absorbance maximum (ϵ_{282}, 10 700 M^{-1} cm^{-1} at pH 6.8). Because diaminouracil was such a slow substrate, large amounts of enzyme were required, and so the assays were conducted in a microvolume cuvette in a total volume of 120 μL. The pH dependence of the catalytic reaction was determined using the following buffers: 100 mM MES (pH 5.5−6.5); 100 mM TES (pH 6.5−8.0); 100 mM CHES (pH 8.5−10.0); and 100 mM CAPS (pH 10.5−11.0). For each assay, the rate of background hydrolysis of diaminouracil was determined and subtracted from the rate measured in the presence of HIUHase. Initial velocity kinetic data were fitted to the Michaelis−Menten equation, and the pH dependence of the kinetic parameters V and V/K were fitted to

$$Y = \frac{C}{(1 + 10^{(pK_1 - pH)})(1 + 10^{(pK_2 - pH)}) + 10^{(pH - pK_3)}} \quad (1)$$

Eq 1 is a function with two ionizations on the acid side and one ionization on the basic side of the pH profile; Y is the parameter being fitted, and C is its pH-independent value.

RESULTS

HIUHase Purification. Only a small fraction of the HIUHase that was expressed in *E. coli* cells appeared in the

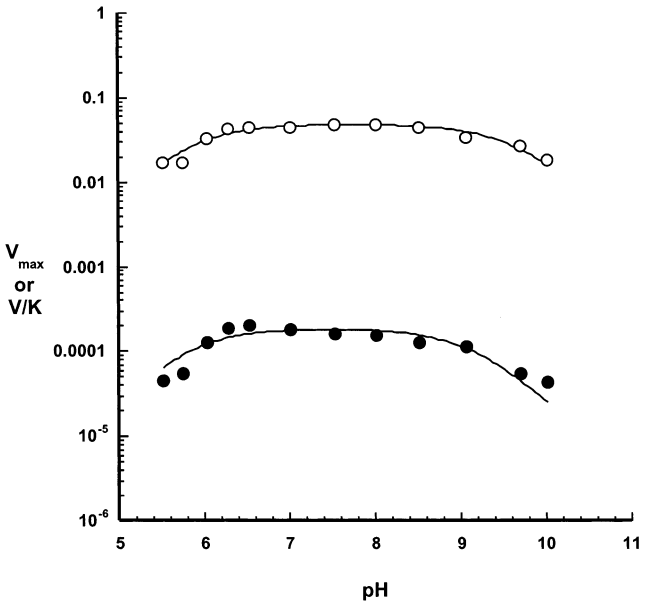


FIGURE 1: V_{max} (○) and V/K (●) as a function of pH for the HIUHase-catalyzed hydrolysis of diaminouracil. The points are experimental, and the lines are the fit to eq 1.

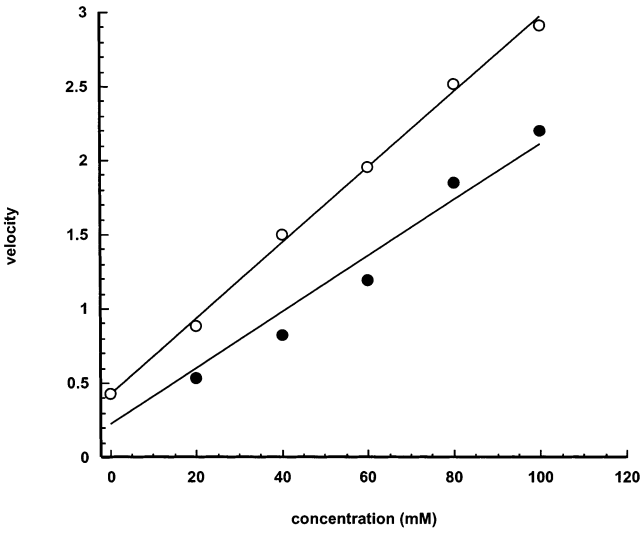


FIGURE 2: Effect of exogenously added nucleophiles to the HIUHase catalyzed hydrolysis of diaminouracil. Azide (○) and alaninamide (●). Diaminouracil was present at 100 μM, and velocity is given in units of μmol/min/μmol HIUHase.

soluble fraction of the cell-free extract. However, the insoluble, His-tagged HIUHase could be purified under denaturing conditions on a Ni^{2+}-affinity column. HIUHase was refolded using conditions identified with a commercial refolding screen. The yield of protein recovered from the refolding procedure varied from 12 to 50%, but the refolded protein had the same specific activity as HIUHase purified from the soluble portion of the cell-free extract. The protein used in the experiments described below was pure, based on the criterion of Coomassie-stained SDS−polyacrylamide gels.

Kinetics with Diaminouracil. Diaminouracil was identified as an alternative substrate for HIUHase. A plot of the absorbance maximum of diaminouracil versus pH yielded a titration curve from which a pK of 4.6 ± 0.3 was calculated (data not shown). This value is in reasonable agreement with the value of 4.3 obtained by potentiometric titration (*9*). The K_m for diaminouracil was 210 ± 5 μM, and the turnover number was 1.6 min^{-1}. These values are to be compared with the K_m for the natural substrate, 5-hydroxyisourate, of 15 μM and a turnover number of 660 min^{-1} (*6*). The kinetic parameters V_{max} and V/K were determined as a function of pH using diaminouracil and are shown in Figure 1. Both parameters described bell-shaped curves with two ionizations on the acid side and one ionization on the basic side. We were unable to obtain meaningful fits when all three pKs were treated as parameters to be determined by the fit. This situation is not uncommon; the fitting algorithm is able to determine the sum of the pKs well but not their individual values. Therefore, we fixed the value of one pK at 4.6, the pK of diaminouracil, and determined the values of the other two pKs in the V/K plot to be 5.7 ± 0.2 and 9.2 ± 0.2 and in the V_{max} plot to be 5.8 ± 0.1 and 9.7 ± 0.1.

Kinetic Properties of HIUHase Mutants. The E199A mutant of HIUHase had so little catalytic activity that V_{max} and K_m could not be determined. However, at 100 μM diaminouracil, the rate of the reaction was 0.0017 μmol/min/μmol HIUHase; under the same conditions the rate of the reaction catalyzed by wild-type enzyme was 0.43 μmol/min/μmol HIUHase. No catalytic activity could be detected with the E408A mutant of HIUHase. To ensure that artifacts were not introduced by the refolding procedure, the small fraction of each expressed mutant protein that was soluble was purified and characterized. For the E199A mutant, the activity of the soluble protein matched that of the refolded protein; the soluble E408A mutant had no detectable activity.

Effect of Exogenous Nucleophiles. The HIUHase-catalyzed hydrolysis of 100 μM diaminouracil was tested at pH 6.8 in the presence of the nucleophilic reagents alaninamide, azide, and hydroxylamine. The rate of the control reaction (no nucleophile added) was 0.4 μmol/min/μmol HIUHase; in the presence of 100 mM hydroxylamine, alaninamide, and azide, the rates were 0.8, 2.2, and 3.2 μmol/min/μmol HIUHase, respectively. The concentration dependence of the reactions in the presence of azide and alaninamide are shown in Figure 2. The E199A and E408A mutants were also tested in the presence of azide. No catalytic activity could be detected with the E408A mutant; the azide concentration dependence of the E199A-catalyzed reaction is shown in Figure 3.

DISCUSSION

HIUHase participates in the ureide pathway in soybeans to catalyze the hydrolysis of the unstable metabolite 5-hydroxyisourate (*6*, *7*). The sequence of the gene encoding HIUHase reveals considerable homology to Family 1 retaining glycosidases (*8*); it is 33% identical to maize β-glucosidase isozyme 1 (GenBank accession no. U25157) and 57% identical to a putative glycosidase in *Arabidopsis* (GenBank accession no. AAF02882). In particular, the sequence suggests that two glutamate residues, Glu 199 and Glu 408, are at the active site in HIUHase. In retaining glycosidases, the analogous residues play critical roles in the catalytic reaction. One Glu residue acts as a nucleophile toward the anomeric carbon of the substrate, displacing the leaving group, and forming a covalent intermediate. The second Glu provides general acid catalysis to assist in the departure of the leaving group, then abstracts a proton from water to activate it for hydrolysis of the covalent intermediate (*10*).

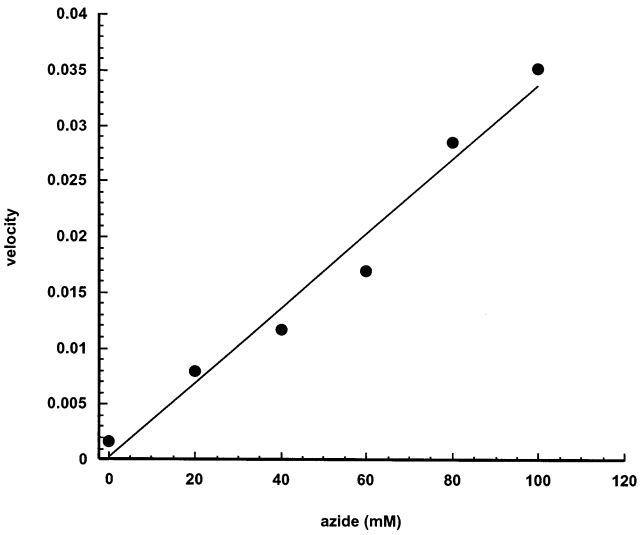
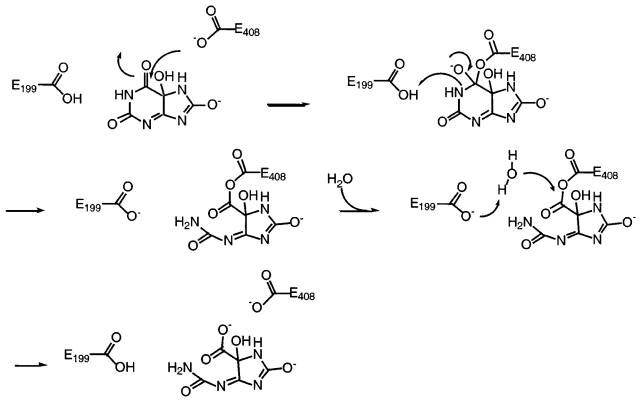


FIGURE 3: Effect of azide on the rate of hydrolysis of diaminouracil catalyzed by the HIUHase E199A mutant. Diaminouracil was present at 100 μM, and velocity is given in units of μmol/min/μmol HIUHase.

Scheme 2



Scheme 2 illustrates a potential mechanism for the hydroxyisourate hydrolase reaction that is drawn in analogy with the mechanism of retaining glycosidases. Several predictions can be made based on the proposed mechanism. First, the catalytic activity of mutant enzymes in which either one of the Glu residues is mutated should be severely compromised. Second, the active site Glu residues should be in opposite protonation states at the beginning of the catalytic cycle, and if their p*K*s fall within the experimentally accessible range, the pH dependence of the kinetic parameters should reflect the protonation states they adopt for catalysis. Third, a covalent enzyme−substrate anhydride is predicted to occur as an intermediate. If hydrolysis of the covalent intermediate is rate-limiting in the overall reaction, addition of a nucleophilic reagent such as azide may increase the rate of the reaction by outcompeting water for reaction with the anhydride intermediate.

A key mechanistic question is whether a covalent intermediate forms during the catalytic reaction since one could also envision a reasonable mechanism for the HIUHase reaction in which the roles of the Glu residues are analogous to the Asp residues in aspartic proteases. HIV protease is an exemplar of this enzyme family, and elegant studies have established that the active site Asp's act as general acid and general base to activate H_2O and mediate the complex series of proton transfers that are required for amide bond hydrolysis without the formation of a covalent enzyme−substrate intermediate (*11*).

To date, detailed mechanistic studies of HIUHase have been precluded by the technical difficulties inherent in working with the unstable substrate. 5-Hydroxyisourate has a half-life of about 20 min at room temperature and for in vitro characterization is generated in situ from urate using urate oxidase. To facilitate further studies of the catalytic mechanism of HIUHase, we sought an alternative substrate. Diaminouracil proved to be a slow substrate for HIUHase, and although it does undergo spontaneous hydrolysis, particularly at high pH, it is considerably more stable than 5-hydroxyisourate and has the further virtue of being commercially available.

We have previously reported that the HIUHase E199A and E408A mutant proteins lacked detectable catalytic activity in reactions using 5-hydroxyisourate hydrolase as substrate. However, because of the rapid nonenzymatic decomposition of 5-hydroxyisourate, it was difficult to detect low levels of activity, and we were able to conclude only that the activity of the mutant enzymes was at least an order of magnitude lower than wild-type enzyme (*7*). Using diaminouracil as a substrate, we have been able to characterize the E199A and E408A mutants more rigorously; still, the activity was too low to determine kinetic parameters. At a single concentration of diaminouracil (100 μM, i.e., below its K_m), the activity of the E199A protein was 0.4% that of wild type. Under the same conditions, no activity could be detected with the E408A mutant. Thus, it is clear that Glu 199 and Glu 408 play critical roles in the HIUHase reaction.

The pH profiles for HIUHase-catalyzed hydrolysis of diaminouracil are shown in Figure 1. Both V_{max} and V/K describe bell-shaped curves; because diaminouracil is a slow substrate, it is likely that the p*K*s that are observed are true thermodynamic p*K*s. Two ionizations are evident on the acid side of the profiles. One ionization can be assigned to one of the amino groups of diaminouracil (*9*). The other two p*K*s must arise from ionizations of functional groups on the enzyme, and the Glu residues at the active site are attractive candidates. If this assignment is correct, it suggests that the Glu residues do indeed exist in opposite protonation states as shown in Scheme 1. The kinetic data do not allow for unambiguous assignment of the ionizing residues, and the assignment of a p*K* above 9 to a glutamate residue warrants scrutiny. However, we note that NMR measurements have directly established the ionization of a glutamate residue with a p*K* around 9 in a mutant xylanase in which a third glutamate residue was introduced into the active site (*12*). Thus, it is clear that electrostatic interactions between ionizing residues can be sufficient to perturb the p*K*s of Glu residues by several pH units. Furthermore, Glu 170, which has been assigned the role as acid/base catalyst in *Agrobacterium* β-glucosidase, has a p*K* of 8.1 (*13*). Because the HIUHase pH profiles show the p*K*s in the V_{max} profile as well as the V/K profile, it appears that diaminouracil can bind to the enzyme when the ionizable groups on the enzyme or substrate are in the incorrect protonation state for catalysis (*14*).

A classic test for the intermediacy of acyl-enzyme species in protease reactions is to run the reaction in the presence of an additional nucleophile; if deacylation is rate-limiting,

the added nucleophile can accelerate the rate of the reaction by competing successfully with H_2O for reaction with the intermediate (*15*). Withers and Zechel have shown that a similar approach can be used to evaluate the intermediacy of covalent substrate−enzyme adducts in reactions catalyzed by retaining glycosidases (*16*). In the present case, addition of azide, alaninamide, and hydroxylamine all increased the rate of hydrolysis of diaminouracil. This experiment is particularly helpful for diagnosing the formation of a covalent intermediate because stopped-flow spectroscopic experiments are precluded by the high K_m for diaminouracil and the fact that its absorption spectrum largely overlaps that of the protein.

Furthermore, the roles of the active site Glu's can be addressed, based on the effects of added nucleophiles to the mutant enzyme reactions. The rate of the reaction catalyzed by the E199A mutant was stimulated over 20-fold by the addition of 100 mM azide. In contrast, the E408A protein, which showed no activity in the absence of exogenous nucleophiles, was still completely inactive in the presence of 100 mM azide. Thus, azide is able to replace Glu 199 but not Glu 408. These data suggest that Glu 408 acts as the active site nuclophile to form the anhydride intermediate with the substrate and that one of the functions of Glu 199 is to serve as the general base to activate H_2O for hydrolysis of the intermediate. In the E199A protein, the role of the general base catalyst is replaced by the reaction of the anhydride intermediate with the anionic nucleophile azide. However, azide apparently cannot replace the presumptive function of the carboxyl group of Glu 408, namely, to form the enzyme−substrate covalent intermediate, so addition of azide to the E408A reaction has no effect. Although mutational analyses of retaining glycosidases have established that the nucleophilic residue can be replaced functionally by an anionic nucleophile such as azide, the replacement is successful only when the substrate contains an activated leaving group (*17*). In the present case, addition of a nucleophile to the amide functionality of the substrate is expected to be more difficult than addition to the anhydride intermediate, so the functional replacement of Glu 199 by azide, and failure of azide to replace Glu 408, is consistent with the assignment of Glu 408 to the role as active site nucleophile and Glu 199 to the role of acid/base catalyst (Scheme 1). Furthermore, pairwise alignment of the HIUHase sequence with *Agrobacterium* β-glucosidase reveals that HIUHase Glu 199 aligns with Abg Glu 170, which has been assigned the role of acid/base catalyst, and HIUHase Glu 408 aligns with Abg Glu 358, which acts as the nucleophile (*17*).

Thus, the weight of the evidence supports the conclusion that the putative active site glutamate residues, Glu 199 and Glu 408, act in the HIUHase reaction in a manner analogous to the active site Glu's in retaining glycosidases, and the roles of Glu 199 and Glu 408 have been assigned as shown in Scheme 1 based on the effect of azide on the reactions catalyzed by the mutant proteins.

Hydrolysis of amide bonds is, of course, a common reaction in metabolism, and many catalytic motifs have been employed to accomplish that chemistry. On the basis of substrate structure, it might have been reasonable to expect that the enzyme responsible for the hydrolysis of HIU would be related to dihydroorotase, which catalyzes the hydrolysis of an amide bond in a pyrimidine substrate. However, dihydroorotase is a member of the amidohydrolase superfamily, and substrate hydrolysis is mediated by a binuclear zinc cluster (*18*). Thus, consideration of the structure of HIU and its metabolic fate would not have pointed toward HIUHase as the enzyme mediating that transformation. Similarly, it is unlikely that examination of the sequence of the gene encoding HIUHase would have identified its metabolic role since it shows so many similarities to retaining glycosidases. Therefore, the identification and characterization of HIUHase may be considered a cautionary tale that illustrates the potential limitations of genomic approaches and the importance of direct methods in the discovery of novel catalysts and metabolic pathways.

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