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Acid–Base Catalytic Mechanism of Dihydropyrimidinase from pH Studies†

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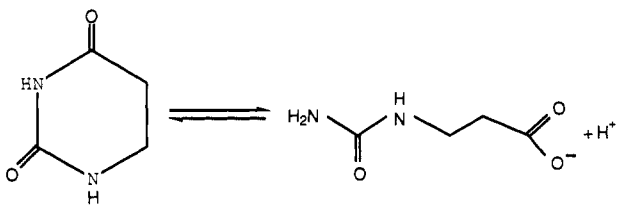
ABSTRACT: The pH dependence of kinetic parameters and solvent deuterium isotope effects have been used to probe the mechanism of the dihydropyrimidinase from the liver of pig and calf. The V/K for 5,6-dihydrouracil (DHU) (or the alternative substrate glutarimide) measured with either the native zinc or cadmium-substituted enzymes decreases at both low and high pH giving pK values of about 7.5–8 and 9–10. The low pK value observed in V is perturbed significantly to lower pH (~6), and the high pK is not observed. The binding of glutarate monoamide is optimum when the group with a pK of 7.7 is protonated, and this same group must be protonated for the reverse reaction, that is, formation of DHU from *N*-carbamoyl-β-alanine. These data are consistent with a general base mechanism and in addition suggest that the enzyme is present initially with a water bound to the active site zinc. The enzymic general base with a pK of 7.5–8 is required to activate water for nucleophilic attack on the C-4 of 5,6-dihydrouracil which is directly coordinated to the active site zinc. The second group with a pK of 9–10 likely reflects Zn–water ionization of the free enzyme. The water bound to the active site Zn is displaced by reactant binding, and thus the pK of 9–10 is not observed in the V profile. Solvent deuterium isotope effects are near unity on the V/K for the natural substrate 5,6-dihydrouracil, but a finite effect of 1.6 is observed on V. Data suggest a rate-determining transition state under V/K conditions for which no protons are in flight. Some transition state after hydrolysis of the amide bond for which one or more protons are in flight likely limits under V conditions. The latter solvent-sensitive step likely includes the deprotonation of the resulting carboxylic acid product and its subsequent release.

5,6-Dihydropyrimidine amidohydrolase (dihydropyrimidinase) (EC 3.5.2.2) is the second enzyme in the pyrimidine degradative pathway catalyzing the reversible hydrolysis of 5,6-dihydrouracil to *N*-carbamoyl-β-alanine (Scheme I). Pyrimidine degradation is important for the following reasons. First, degradation of uracil via 5,6-dihydrouracil and *N*-carbamoyl-β-alanine is the only biosynthetic pathway leading to β-alanine in mammals (Wasternack, 1978), whereas procaryotic cells produce β-alanine by α-decarboxylation of aspartate (Williamson & Brown, 1979). Several lines of evidence indicate that β-alanine has putative neurotransmitter qualities (Sandberg & Jacobson, 1981; Toggenburger et al., 1982). Second, degradation of pyrimidines is responsible for the catabolism of clinically applied pyrimidines, such as 5-fluorouracil, in tumor treatment (Naguib et al., 1985; Tuchman et al., 1985; Hull et al., 1988). Dihydropyrimidinase has been found to participate in the action of IRCF-187 [1,2-bis(3,5-dioxopiperazinyl-1-yl)propane] by catalyzing its ring opening to provide a chelator and reducing the toxicity of iron bound to adriamycin (Hasinoff et al., 1991). Third, neurological abnormalities were reported to develop in patients with familial pyrimidinemia and pyrimidinuria when treated with 5-fluorouracil (Tuchman et al., 1985; Diasio et al., 1988).

The dihydropyrimidinase has been found in both kidney and liver of various mammalian systems and was purified to homogeneity from bovine liver (Lee et al., 1986) and calf liver

Scheme I: Reaction Catalyzed by Dihydropyrimidinase



(Kautz & Schnackerz, 1989). This enzyme is a tetramer and contains four tightly bound zinc ions (Brooks et al., 1983), which can be removed by chelators such as 8-hydroxyquinoline sulfonic acid and 2,6-dipicolinic acid (Lee et al., 1986). The pH dependencies of V and V/K for the native zinc and manganese-substituted DHPases[1] using 5-bromo-5,6-dihydrouracil as a substrate were found to be similar, showing the requirement for a single group that must be protonated for activity (Lee et al., 1987).

In this report, we have studied the pH dependence of kinetic parameters for the dihydropyrimidinase from the liver of pig and calf to establish a working catalytic mechanism. Furthermore, isotope effects are used to identify the slow step(s) along the reaction pathway.

MATERIALS AND METHODS

Materials. Dihydrouracil, dihydrothymine, *N*-carbamoyl-β-alanine, 8-hydroxyquinoline hemisulfate, and deuterium oxide were purchased from Sigma. Chelating Sepharose 6B,

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[1] Abbreviations: DHPase, dihydropyrimidinase; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTE, dithioerythritol; SDS, sodium dodecyl sulfate; DHU, 5,6-dihydrouracil; GAMA, glutarate monoamide; NCBA, *N*-carbamoyl-β-alanine, GI, glutarimide.

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Table I: Purification of 5,6-Dihydropyrimidine Amidohydrolase from Pig Liver Starting with 20 g of Pig Liver Acetone Powder

purification step	volume (mL)	total protein (mg)	total activity (units)	specific activity (units/mg)	purification (fold)	yield (%)
crude extract	170	4250	19.9	0.0047	1.0	100
heat treatment	158	1359	19.6	0.0144	3.1	99
$(NH_4)_2SO_4$ (35–60%)	11	605	19.6	0.0323	6.9	99
octyl-Sepharose 6B	15	90	10.2	0.113	24.1	51
chelating Sepharose 6B	13	12	8.1	0.675	143.9	41
DEAE-Sepharose 6B	4.7	6.7	7.1	1.06	224.8	35

chelating Sepharose Fast Flow, DEAE Sepharose Fast Flow, octyl Sepharose 6B, and Sepharose G100 were obtained from Pharmacia. Cadmium chloride was from Aldrich. All other reagents were of the highest purity available from commercial sources.

Enzyme Assays. Dihydropyrimidinase activity was assayed by measuring spectrophotometrically the change in absorbance at 225 nm caused by hydrolysis or formation of the 5,6-dihydropyrimidine ring using either a Pye Unicam SP8-100 or a Cary model 3 recording spectrophotometer thermostated at 37 °C. The molar absorption coefficients at 225 nm for DHU and glutarimide are 1287 and 400 M^{-1} cm^{-1}, respectively. For routine enzyme assays, reaction mixtures containing 0.1 M potassium phosphate, pH 8.0, and 0.25 mM DHU were used. One unit of DHPase catalyzes the hydrolysis of 1 μmol DHU/min at 37 °C under the above conditions. For pH studies the following buffers were used over the pH range indicated: potassium phosphate, 6–8; potassium pyrophosphate, 8–10. Protein concentrations were determined by the method of Schaffner and Weissmann (1973).

Enzyme Purification. Dihydropyrimidinase was purified from pig liver acetone powders by extracting the powder 2.5 times with five volumes of 10 mM potassium phosphate, pH 7.0, heat treatment (5 min at 58 °C), and ammonium sulfate fractionation (35–60% saturation at pH 7.0). After dialysis against 10 mM potassium phosphate, pH 7.0, containing 0.5 mM DTE and 1.5 M ammonium sulfate, the enzyme was applied to an octyl Sepharose 6B column (1.6 × 40 cm) equilibrated with dialysis buffer, and DHPase was eluted with a 1.5–0 linear ammonium sulfate gradient in 10 mM potassium phosphate, 0.5 mM DTE, pH 7. The pooled DHPase fractions were concentrated by ultrafiltration using an Amicon PM-10 diaflo filter. The enzyme was then dialyzed against 20 mM potassium phosphate, pH 7, containing 0.5 mM DTE and 0.5 M KCl, and applied to a chelating Sepharose 6B column (1 × 20 cm) loaded with zinc ions. The DHPase was eluted with a linear 0–1 M glycine gradient in the equilibration buffer. The DHPase fractions were concentrated by ultrafiltration and then dialyzed against two 500-mL changes of 50 mM Tris-HCl, pH 8.5, containing 0.5 M KCl and 0.5 mM DTE. The dialyzed enzyme was then applied to a DEAE Sepharose 6B column (1.6 × 20 cm). Elution was carried out using a linear gradient of 0–0.2 M KCl in the dialysis buffer (2 × 200 mL). The pooled DHPase fractions were combined and concentrated by ultrafiltration as described above, to a final protein concentration of about 3 mg/mL, and stored at 4 °C.

The DHPase was also purified from calf liver using a procedure improved compared to that described above. An acetone powder was prepared from 10–20 g of calf liver and extracted twice with five volumes of 10 mM potassium phosphate, pH 7, by stirring and using a mortar and pestle at room temperature. The preparation was then centrifuged at 13000*g*. The supernatant was then subjected to a heat treatment (5 min at 62 °C) and centrifuged as above. Ammonium sulfate was then added to the supernatant to 60% saturation (pH 7.5 and 4 °C), the pellet was dissolved in 20 mM potassium phosphate, pH 7, and dialyzed against the same buffer. The dialysate was fractionated on a chelating Sepharose Fast Flow column (1.6 × 15 cm) equilibrated with the dialysis buffer. After washing, the column was developed using a discontinuous glycine gradient from 0 to 1 M with DHPase eluting between 0.3 and 0.8 M glycine. The active fractions were pooled and concentrated using Amicon PM 10 ultrafiltration and then dialyzed against 50 mM Tris-HCl, pH 8.5. The dialysate was then subjected to chromatography on a DEAE Sepharose Fast Flow column (1 × 10 cm) equilibrated with dialysis buffer and developed using a 200-mL gradient of 0–0.15 M KCl in equilibration buffer. Active DHPase fractions were combined and concentrated by ultrafiltration as described above and chromatographed on a Sephadex G100 column (1.6 × 90 cm). The DHPase fractions were homogeneous by the criterion of SDS–PAGE.

Preparation of Apoenzyme and Reconstitution with Cd^{2+}. Apoenzyme was prepared by dialyzing DHPase at a concentration of 0.2 mg/mL against 200 volumes of 5 mM 8-hydroxyquinoline hemisulfate at pH 7 for 160–200 h at 37 °C to remove the active site zinc. To reconstitute the apoenzyme with Cd^{2+}, 8-hydroxyquinoline is removed from the solution by dialysis against metal-free water followed successively by dialyses against 200 volumes of 50 μM $Cd(Cl)_2$ in water for 4 h and against 500 volumes of water for 30 min. The latter were carried out at room temperature. Finally, the reconstituted Cd–enzyme was dialyzed against 10 mM potassium phosphate, pH 8, at 4 °C.

Zinc and Cadmium Determinations. The zinc and cadmium content was determined by atomic absorption spectroscopy (Perkin-Elmer model 3030). Samples were prepared by dialysis against 10 mM potassium phosphate, pH 7.0, or 10 mM potassium phosphate and 50 mM EDTA, pH 7.0. Metal-free stock solutions according to Himmelhoch et al. (1971) were used. In all cases the stoichiometry of metal/enzyme subunits was within error 1.

Determination of Kinetic Isotope Effects. For solvent isotope effects all reactants were prepared in D_2O, and pD-rate profiles were obtained. The ratio of the pH(D) independent values of the parameter of interest gives the solvent deuterium isotope effect. All buffers and reactant solutions prepared in D_2O were adjusted to the appropriate pD with KOD or DCl. The nomenclature is that of Northrop (1977) as modified by Cook and Cleland (1981) where the leading superscript depicts the isotope effect on the parameter of interest.

Data Processing. Reciprocal initial velocity was plotted versus reciprocal substrate concentrations, and experimental data were fitted by a nonlinear least-squares method and the Fortran programs of Cleland (1979). pH profiles decreasing at low and high pH with linear slopes of +1 and −1, at low pH with a slope of +1 or a high pH with a slope of −1, were fitted using eqs 1–3. Data decreasing from a constant value at low pH to another constant value at high were fitted using eq 4. Data conforming to linear competitive or noncompetitive inhibition were fitted using eqs 5 and 6. In eqs 1–4, Y is the

$$\log Y = \log[C/(1 + H/K_1 + K_2/H)] \quad (1)$$

$$\log Y = \log[C/(1 + H/K_1)] \quad (2)$$

$$\log Y = \log[C/(1 + K_2/H)] \quad (3)$$

$$\log Y = \log[Y_L + Y_H(K_1/H)/(1 + K_1/H)] \quad (4)$$

$$v = VA/[K_a(1 + I/K_{is}) + A] \quad (5)$$

$$v = VA/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (6)$$

value of the parameter of interest measured at any pH, C is the pH independent value of Y, H is the concentration of H^+, and K_1 and K_2 represent dissociation constants for enzyme groups, while Y_L and Y_H represent the pH independent values of $1/K_{i\,GAMA}$ at low and high pH, respectively. In eqs 5 and 6, v is initial velocity, V is maximum velocity, and K_a is the K_m value for A, while K_{is} and K_{ii} are the inhibition constants for I on slope and intercept, respectively.

RESULTS

Purification and Properties of Dihydropyrimidinase. A typical purification procedure for DHPase from acetone powders of pig liver is summarized in Table I. The purification steps used are very similar to those for the calf liver enzyme (Kautz & Schnackerz, 1989). The chelating and ion exchange chromatography constituted the best purification steps. The DHPase was purified 225-fold with a specific activity of 1.1 units/mg of protein and a recovery of 35%. The subunit molecular mass of the pig liver DHPase determined by SDS–PAGE was found to be 54 kDa, whereas the molecular mass of the native enzyme is 210 kDa (data not shown). The improved purification of the calf liver enzyme gave a final yield of 67% with a specific activity of 7.5 units/mg.

pH Dependence of Kinetic Parameters and Solvent Deuterium Isotope Effects. Using DHU as a substrate for the pig liver enzyme, the maximum velocity decreases at low pH giving a pK of about 6, while the V/K_{DHU} decreases at low and high pH giving pK values of about 8 and 9 (Figure 1). A repeat of the above experiments in D_2O gave a pK value of 7 for V and pK values of 8.6 and about 10 for V/K. The pH-independent values in H_2O of V and V/K are 0.85 ± 0.05 s^{-1} and (1.29 ± 0.02) × 10^5 M^{-1} s^{-1}, respectively. The solvent isotope effect calculated as the ratio of the pH-independent values in H_2O and D_2O are as follows: ^{D_2O}V = 1.6 ± 0.1; $^{D_2O}(V/K_{DHU})$ = 1.4 ± 0.4. To determine whether there was any difference between the pig and calf liver enzymes, the above experiments in water were repeated with the calf liver enzyme (Figure 2). The V is pH independent between 6.5 and 10, in agreement with the data discussed above, while the V/K for DHU decreases at low and high pH giving pK values of about 7.5 and 10.2 (but the latter has a large standard error). The pH-independent values of V and V/K are 9 ± 1 s^{-1} and (3.1 ± 0.4) × 10^5 M^{-1} s^{-1}, respectively. Thus, the V/K is identical to that of the pig liver enzyme, while the V is about 10-fold higher. The latter may be in part due to the improved procedure for preparation of the calf liver enzyme. The pK values estimated from the pH dependence of kinetic parameters are summarized in Table II.

Glutarimide (GI) is also a substrate for the DHPase with a V 20% that of DHU (Kautz & Schnackerz, 1989). The pH dependence of kinetic parameters obtained with GI and the calf liver enzyme are qualitatively identical to those obtained with DHU (data not shown). The V decreases at low pH with a pK of 7.2, while V/K_{GI} decreases at low pH giving a pK value of about 8. The pH-independent values of V and V/K are 7.5 ± 1.4 s^{-1} and (1.9 ± 0.3) × 10^4 M^{-1} s^{-1}, respectively.

Replacement of the active site Zn^{2+} with Cd^{2+} results in an enzyme that is substantially more active (Kautz & Schnackerz, 1989). The pH dependence of kinetic parameters and solvent isotope effects were measured using the Cd-substituted calf liver enzyme. Data are again qualitatively

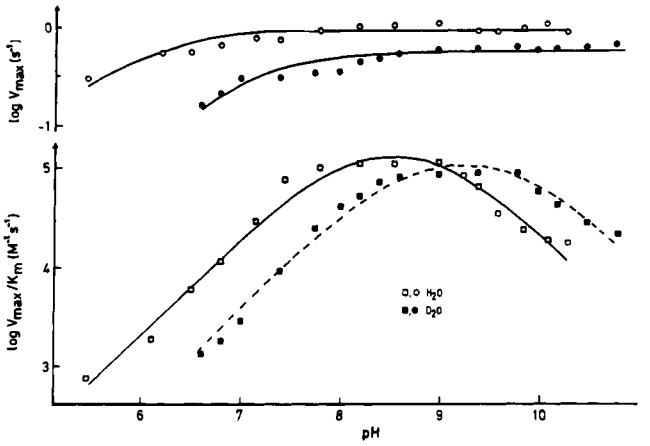


FIGURE 1: pH dependence of the V and V/K in H_2O and D_2O for the pig liver enzyme using DHU as the substrate. All assays were carried out at 37 °C with a DHPase concentration of 16.35 μg/mL. Points are experimental values, while the curves are from a fit using eq 1 for V/K and eq 2 for V. Open and closed symbols denote experiments carried out in H_2O and D_2O, respectively.

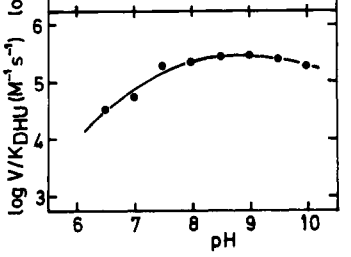


FIGURE 2: pH dependence of V and V/K_{DHU} for the calf liver enzyme using DHU as the substrate. All assays were carried out at 37 °C with a DHPase concentration of 16.35 μg/mL. Points are experimental values, while the curve for V/K is from a fit using eq 1 and that for V represents an average value.

Table II: Summary of pK Values from the pH Dependence of Kinetic Parameters

parameter	pK_a	pK_b
	Native	
V_{DHU}	5.9 ± 0.1[a] (7.0 ± 0.1)[b]	
V_{GI}	7.2 ± 0.2	
V/K_{DHU}	8.0 ± 0.3 (8.6 ± 0.2)	9.0 ± 0.4 (9.8 ± 0.3)
V/K_{GI}	8.2 ± 0.2	
$1/K_{i\,GAMA}$	7.7 ± 0.1	
	8.3 ± 0.1[c]	
V/K_{NCBA}[d]	7.6 ± 0.1	
	Cd–Enzyme	
V_{DHU}	7.1 ± 0.2 (7.6 ± 0.2)	
V/K_{DHU}	7.3 ± 0.2 (7.9 ± 0.3)	10.2 ± 0.3 (11.4 ± 0.6)

[a] In the presence of 0.25 mM GAMA, the pK is 6.3 ± 0.1. [b] pK values in parentheses are obtained in D_2O. [c] The pK of 7.7 is perturbed to 8.3 when GAMA binds. [d] The V/K for *N*-carbamoyl-β-alanine in the direction of DHU synthesis.

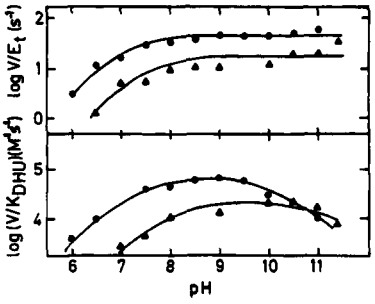


FIGURE 3: pH dependence of kinetic parameters for the Cd-substituted calf liver enzyme in H_2O and D_2O using DHU as the substrate. Conditions are as in Figure 1. Closed circles and triangles represent experiments carried out in H_2O and D_2O, respectively.

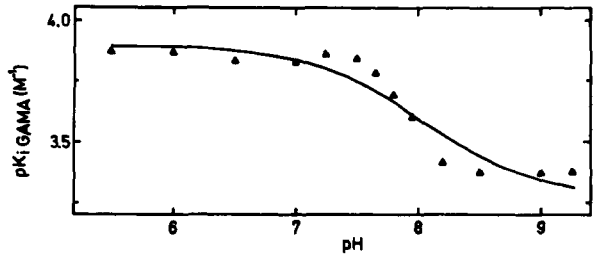


FIGURE 4: pH dependence of $1/K_i$ for GAMA for the pig liver enzyme. The K_i for GAMA was obtained at each pH from full inhibition profiles varying DHU at different levels of GAMA. The points are experimental, and the curve is from a fit of the data using eq 4.

similar to those of the Zn–enzyme with DHU as a substrate. The V decreases at low pH giving a pK of 7.1, while V/K decreases at low and high pH giving pK values of 7.3 and 10.2, respectively (Figure 3). The pH-independent values of V and V/K are 51 ± 4 s^{-1} and (6.2 ± 0.7) × 10^4 M^{-1} s^{-1}, respectively, while the solvent isotope effects on V and V/K are 2.6 ± 0.5 and 4.0 ± 1.5, respectively.

Finally, the pH dependence of the V/K for the product N-carbamoyl-β-alanine in the direction of DHU synthesis was also measured (data not shown). The V/K decreases at high pH with a pK of 7.6 and gives a pH-independent value of 150 ± 20 M^{-1} s^{-1}.

Inhibition Data. A number of analogs compete with DHU for the active site of DHPase, including the products of the reactions with DHU (NCBA) and GI (GAMA). Inhibition patterns using these products and other dead-end analogs provide the data in Table III. The pH dependence of $1/K_{i\ GAMA}$ was also obtained using the pig liver enzyme (Figure 4). The pK_i is constant at low pH and decreases above a pK of 7.7 to a new constant value at higher pH with a pK of 8.3. The pH-independent values of $1/K_{i\ GAMA}$ at low and high pH are included in Table III.

DISCUSSION

Interpretation of pH Profiles. The pK values observed in the V and V/K pH-rate profiles reflect enzyme residues rather than substrate since dihydrouracil has no pK values in the range 5–10. The V/K_{DHU} profile reflects pK values in free enzyme, and the observed pH dependence indicates that two groups are required for catalysis and/or binding, one protonated and another unprotonated. Qualitatively similar results are observed for V/K_{GI}, suggesting that the same residues are involved in hydrolysis of this alternative substrate. One of the groups is likely a general base, but it is not possible to determine which of the two pK values represents the general base from only the V/K pH-rate profiles. The V/K_{NCBA} obtained in the direction of DHU formation decreases as the

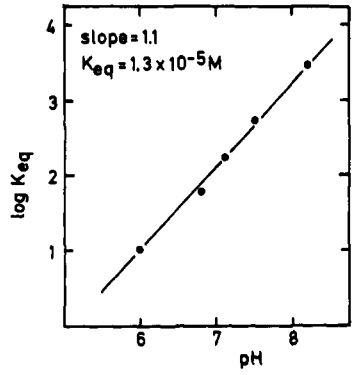


FIGURE 5: pH dependence of K_{eq} was calculated as the ratio $(V/K_{DHU})/(V/K_{NCBA})$ at each of the pH values indicated using the experimental points obtained in Figures 1 and 4. The slope of the curve is calculated using a linear regression.

Table III: Inhibition Studies of Dihydropyrimidine Amidohydrolase in 100 mM Potassium Phosphate, pH 8.0, at 37 °C

inhibitor		K_i (mM)
N-carbamoyl-β-alanine	H_2N-CO-NH-CH_2-CH_2-COO^-	0.68 ± 0.04
glutaric acid monoamide	H_2N-CO-CH_2-CH_2-COO^-	0.21[a]
4-ureidobutyric acid	H_2N-CO-NH-CH_2-CH_2-CH_2-COO^-	0.99 ● 0.06
5-amino-n-valeric acid	H_2N-CH_2-CH_2-CH_2-CH_2-COO^-	7.3[b]
propionic acid	CH_3-CH_2-COO^-	11.8[b]
propionic acid amide	^+H_3N-CO-CH_2-CH_3	17.0[b]
β-alanine	^+H_3N-CH_2-CH_2-COO^-	31.8[b]

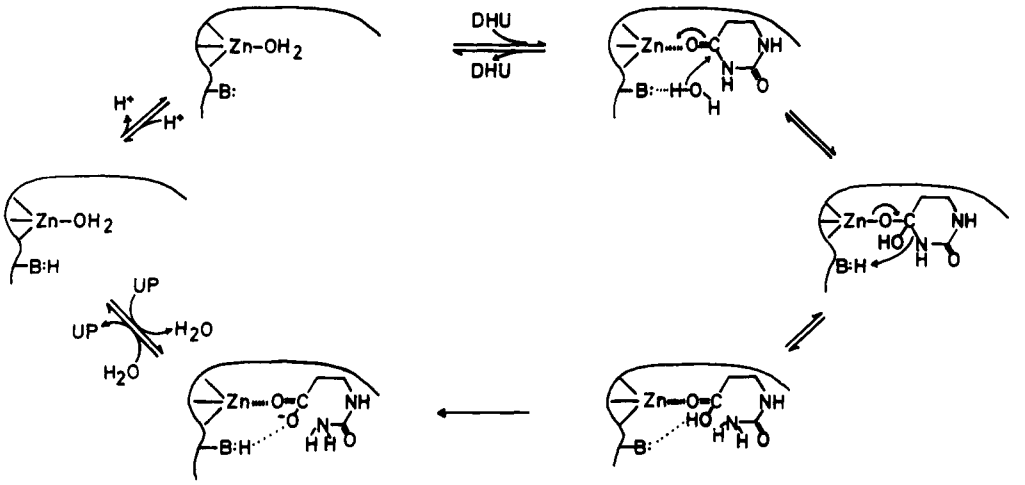
[a] The pH-independent value of $K_{i\ GAMA}$ at low pH is 126 ± 5 μM while that at high pH is 530 ● 30 μM. [b] Determined by graphical analysis.

pH is increased giving a pK of 7.6, within error identical to that observed on the acid side of the V/K profiles for DHU and GI. Thus, the group with a pK of 7.5–8 must be protonated in the direction of synthesis of DHU and unprotonated in the direction of DHU hydrolysis (see mechanism below). A change in the tightly bound active site divalent metal ion to Cd^{2+} (Kautz & Schnackerz, 1989) gives results that are qualitatively identical to those observed for V/K_{DHU} with Zn^{2+}. There is a quantitative change in that the group with the pK of 7.5–8 in the Zn–enzyme is slightly lower with a pK of 7.3 in the Cd–enzyme. These data may indicate a close proximity of the metal to the general base.

The pK_i profile for GAMA decreases as the pH is increased, giving a pK of 7.7 for a residue in free enzyme required protonated for optimum binding. The identity of the pK value to that of the enzymic general base (vide supra) suggests that it is the latter that must be protonated. The pK_i profile decreases to a constant value at high pH giving a pK of 8.3, indicating that the pK of the general base is perturbed 0.6 pH units upon binding of GAMA (Cleland, 1977), perhaps as a result of a hydrogen bond formed between the two.

The pH dependence of V exhibits a single pK as the pH decreases. However, the pK is displaced to lower pH by approximately 1.5–2 pH units compared to those of 7.5–8 observed in the V/K pH profiles. The observed pK of 5.9 likely reflects the same group that has a pK of 7.5–8 in the V/K profiles, but with a much lower pK. The pK of 7 observed in the V profile with GI as a substrate is perturbed only slightly to lower pH compared to a value of 7.5–8. The low pK in the V profile for the Cd–enzyme is 7, identical to that observed in the V/K_{DHU} profile. The possible reasons for perturbation of the pK upon substrate binding include a direct interaction

Scheme II: Proposed Chemical Mechanism for Dihydropyrimidinase



with the substrate, placing a cationic acid group such as imidazole in a hydrophobic environment as a result of a conformation change, or a slow step(s) not contained in V/K, that is, a step that occurs after the first irreversible step. Since the perturbation of the V pK is significantly less with GI as a substrate and there is no perturbation at all observed for the Cd–enzyme, it is likely the latter explanation that is correct, the presence of a slow step in V not found in V/K. This aspect will be considered further below.

The basic pK observed in the V/K profiles is not observed in the V profiles. If the high pK is displaced with DHU as a substrate, as it should be if it is present, to an extent similar to the low pK, it would have a value of 11–12, outside the pH range studied. However, the pK of 10 is also not observed with GI used as a substrate or with the Cd–enzyme, even though the low pK is not perturbed to lower pH in either case. The absence of the high pK in the V profile indicates that it likely reflects ionization of the Zn–water in the free enzyme (Dawson et al., 1979) and that DHU binding displaces the water molecule. In agreement with this suggestion, the pK is higher, as expected for the Cd–enzyme (Lide, 1990). Taken together, the data indicate a single base mechanism with substrate displacing an initially present Zn-bound water.

The equilibrium constant for the DHPase reaction can be calculated from the Haldane relationship (Cleland, 1982). In the case of a uni–uni reaction, the equilibrium constant is the ratio of the V/K values in the forward and reverse reactions, that is, $(V/K_{DHU})/(V/K_{NCBA})$. However, as seen in Scheme I, the K_{eq} is pH dependent. The pH dependence of the equilibrium constant is shown in Figure 5. Note that the log of K_{eq} vs pH has a unit slope indicating the involvement of a single proton, consistent with Scheme I. The calculated average pH-independent equilibrium constant using the values in Figure 5 is 1.3×10^{-5} M.

Interpretation of Inhibition Data. The K_i for NCBA at pH 8 is approximately 600 μM. Replacement of the β-amido with a methylene improves the binding by about 2-fold, suggesting that the presence of the former either interferes with proper binding or that the geometry of the ureido functional group is bound less optimally. The latter would be expected to be somewhat more planar at the ureido group, while the monoamide functional group of GAMA will be likely somewhat less planar at the methylene. Increasing the chain length of the amino acid portion of the molecule weakens the binding slightly. Elimination of the carbonyl of either GAMA or NCBA has a dramatic effect, increasing K_i by an order magnitude. In addition, components such as propionate, its amide, or β-alanine all bind even more poorly. Thus, optimal binding of the product likely requires the proper chain length as provided by β-alanine and a terminal amide.

Interestingly, the V/K for glutarimide is about an order of magnitude less than that obtained for DHU, giving an effect opposite to that discussed above comparing GAMA and β-ureidopropionate. These data may suggest an interaction between an enzyme residue and N1 of DHU. The binding of GAMA may then be as depicted in Scheme II for DHU or with the amide nitrogen of GAMA interacting at the N1 site. Answers to these questions will require further studies of substrate specificity.

Solvent Isotope Effects. Significant solvent isotope effects are observed only on the V_{max} when DHU is used as a substrate for the Zn–enzyme. Although there is an isotope effect of 1.4 $\pm$ 0.4 reported for $^{D_2O}(V/K_{DHU})$, the value is most likely very close to unity. The lack of a finite isotope effect on V/K is best seen by a visual comparison of the data in Figure 1 as compared to those presented for V. The lack of a solvent isotope effect on V/K could be the result of substrate stickiness[2] or the lack of a solvent dependence to the slowest of the steps contained in the rate constant V/K. If the substrate were sticky, the pK observed in the $pK_{i\ GAMA}$ profile would be more basic than that observed in the V/K_{DHU} (or V/K_{GI}) pH profiles. Since the pKs are within experimental error identical, it is unlikey that substrate release from the E·DHU complex limits V/K. Thus, the slow step within V/K must be insensitive to solvent deuterium substitution when Zn^{2+} is the active site metal.

The isotope effect on V suggests a slow step that is not contained in V/K that is sensitive to solvent deuterium substitution. The most likely possibility for this solvent-sensitive step is product release facilitated by general base assistance in deprotonating the resulting carboxylic acid (see below). Although the reaction is not irreversible, opening the ring has an equilibrium constant of 100 at pH 7 which is far toward the ring opened species.[3] As a result, V/K will likely contain all steps up to and including the ring opening, while V contains all steps from the enzyme–substrate complex to dissociation of product. The value of the observed isotope effect is small , with a value of 1.6 suggesting the existence of other slow steps contained in V (perhaps the slow step contained in V/K that will also be seen under V conditions).

[2] A substrate is termed sticky if the partition ratio of the ES complex is toward product compared to unbound species.

[3] Calculated from the ratio of the V/K values obtained at pH 8.

The solvent deuterium isotope effects on V and V/K are 2.6 and 4, respectively, but within error equal when Cd^{2+} is substituted for Zn^{2+} in the active site of DHPase. These data suggest a common rate-determining step that dominates both parameters. The most likely step exhibiting a solvent deuterium effect is that in which water is activated for nucleophilic attack on C-4 of DHU coordinated to the active site metal. Thus, even though the overall turnover number is greater with the Cd–enzyme, it appears that the nucleophilic attack by water is less facile perhaps as a result of the increased size of the divalent metal ion generating some crowding in the active site.

Finally, the pK values calculated from the V and V/K profiles measured in H_2O increase in D_2O as a result of the equilibrium isotope effect on the acid dissociation constant (Quinn & Sutton, 1991). The pK values are expected to increase by 0.4–0.6 pH units in D_2O compared to H_2O for nitrogen- and oxygen-containing functional groups. The latter applies, within experimental error, to the pK increases observed for all of the V/K profiles. The pK calculated from the V profile, however, increases by over a pH unit in D_2O compared to H_2O. This large increase in the pK suggests that the observed pK in H_2O is likely not an intrinsic pK but is perturbed to lower pH as a result of slow step(s) other than those contained within the catalytic pathway. In addition, it is likely that the slow step(s) is(are) sensitive to solvent isotopic composition to account for the large isotope effect on the pK.

Chemical Mechanism. Resting enzyme primed for hydrolysis of DHU has a general base (pK ~7.5–8) and a Zn-bound water (pK ~9–10). Dihydrouracil binds to enzyme displacing the Zn-OH_2 (pK 9.6; Dawson et al., 1979) (Scheme II). Consistent with the assignment of Zn-OH_2, the pK increases to 10.3 as expected when Cd^{2+} is substituted (Lide, 1990). The substrate likely binds with the 4-oxo group directly coordinated to the active site metal, so that the metal acts as a Lewis acid polarizing the carbonyl for the subsequent hydrolysis. The general base activates a water molecule for nucleophilic attack at C-4 to generate a tetrahedral intermediate. This step cannot be rate determining under V/K conditions for the Zn–enzyme, since a solvent isotope effect of near 1 is observed, but is rate determining for the Cd–enzyme since a V/K effect of about 2.6–4 is observed. The tetrahedral intermediate undergoes ring opening assisted by general acid protonation of the ring nitrogen using the same enzyme residue to give *N*-carbamoyl-β-alanine. The ring opening step is likely as suggested above to be irreversible. The general base then accepts a proton from the product to give *N*-carbamoyl-β-alanine, which is then released.

The general base is required protonated in the direction of DHU synthesis as evidenced by the V/K_{NCBA} which decreases as the pH is increased with a pK identical to that observed in the V/K_{DHU} profile. In agreement with the requirement for the protonated general base in the direction of DHU synthesis is the observed optimum binding of GAMA to enzyme in which the group with a pK of 7.7 is protonated.

Comparison to Similar Systems. The effects of pH and inhibitors upon the catalytic activity of dihydroorotase of the multienzyme *pyrl-3* from mouse Ehrlich ascites carcinoma has been studied by Christopherson and Jones (1980). The apparent K_m value for *N*-carbamyl-L-aspartate increases by 2 orders of magnitude as the pH increases from 7.0 to 8.3. This finding has been suggested to be consistent with equilibration of dihydroorotase between four states of protonation ($E \leftrightarrow EH \leftrightarrow EH_2 \leftrightarrow EH_3$), where EH_3 is the only catalytically active form of dihydroorotase for the biosynthetic reaction. However, on the basis of these limited data, it is difficult to determine whether EH, EH_2, or EH_3 is active, particularly since no knowledge is available on the pH dependence of V_{max}. The rapid inactivation of dihydroorotase by L-cysteine and the slower effect of 2-mercaptoacetate is consistent with the time-dependent removal of zinc from the active site. Data on the pH dependencies of the dihydroorotate synthesis and hydrolysis reactions obtained for the *Clostridium oroticum* dihydroorotase are qualitatively similar to those described for mammalian dihydroorotase (Pettigrew et al., 1985). The equilibrium between *N*-carbamyl-L-aspartate and 5,6-dihydroorotate is pH dependent, with the biosynthetic reaction consuming and the degradative reaction generating a hydrogen ion. Consequently, it is to be expected that the biosynthetic reaction is catalyzed by an acidic form of dihydroorotase, while the reverse reaction would be catalyzed by the conjugate base of the same enzymic group. Consistent with this proposal, the V_{max} values for the biosynthetic and the degradative reactions and the apparent K_m values for *N*-carbamyl-L-aspartate show opposite pH dependencies. Pettigrew et al. (1980) suggest that the acidic enzyme residue necessary for binding *N*-carbamoyl-L-aspartate is a Zn(II)-bound water molecule. These authors further suggest that the β-carboxyl group of the substrate may displace the bound water to form an inner sphere coordination complex of substrate with the Zn(II). Ring cleavage of the 5,6-dihydroorotate would be initiated by nucleophilic attack at carbon 4 by OH^- formerly coordinated to Zn(II) (Christopherson & Jones, 1980). These suggestions are in essential agreement with the mechanism proposed in the present study for the dihydropyrimidinase.

The hydrolysis of acetate or *p*-nitrobenzoate esters of mandelic acid by carboxypeptidase is pH dependent, decreasing at low and high pH giving pK values of about 7 and 8 in both V/K and V profiles. The mechanism of carboxypeptidases is thought to proceed via a nucleophilic attack by a water molecule on the carbonyl carbon of the peptide (Coll et al., 1991). The water is thought to be activated by the active site Zn and Glu-270. The pK of 7 likely reflects Glu-270 (Cleland, 1977), while the active site Zn could play a role identical to that proposed for DHPase. Finally, the pH dpendence of the peptide V/K for thermolysin shows two inflection points at 5.9 and 7.5 (Pangburn & Walsh, 1975). Again, it is likely that the low pK reflects an active site general base that activates water, with the active site Zn playing a Lewis acid role in activating reactant. The basic pK has not been identified in the case of either of the proteases.

It appears that Zn-hydrolases have evolved to use a common mechanism. Catalysis is presumably via general base-assisted nucleophilic attack by water and Lewis acid-assisted polarization of the carbonyl at the scissile bond.

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