Rat Liver Imidase*

(Received for publication, December 15, 1992, and in revised form, February 1, 1993)

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Imidase, an enzyme variously identified as dihydropyrimidinase (EC 3.5.2.2), hydantoinase, dihydropyrimidine hydrase, and dihydropyrimidine amidohydrolase, has been purified to electrophoretic homogeneity from rat liver. Although a component in the chain of pyrimidine catabolism, imidase is capable of serving in a broader role that includes detoxication of xenobiotics. The enzyme catalyzes the hydrolytic cleavage of imides that range from the linear to the heterocyclic and that include hydantoins, dihydroimidines, and phthalimide. For some substrates, the reaction is experimentally reversible. The pH activity curves are a function of the pKₐ of the individual substrate’s imino group, with cleavage favored at a pH near the respective pKₐ value. There is evidence for stereoselectivity and for stereospecificity. A mechanism is proposed for the enzyme-catalyzed reaction.

A characteristic of the enzymes of detoxication is their broad specificity for compounds bearing a particular functional group rather than for an individual compound or a type of carbon skeleton (1). Whereas such enzymes can catalyze conversion of normal metabolic intermediates, this function is usually a property of the biosynthetic enzymes, which are endowed with both greater specificity and, frequently, with sensitive regulatory controls. Our search for an enzyme capable of hydrolyzing the wide range of imides that are encountered as xenobiotics disclosed a number of known examples of imidase action, one of which forms part of the salvage pathway for the degradation of pyrimidines (2). An enzyme, originally described as hydantoinase (3), was subsequently recognized as also catalyzing the experimentally reversible hydrolysis of dihydrouracil (Reaction 1) and dihydrothymine (4). It has been obtained in homogeneous form from both Pseudomonas striata (5) and as a zinc metalloprotein from bovine liver (6, 7). The enzyme is presently recorded as dihydropyrimidinase (EC 3.5.2.2) (8). Work with crude enzyme preparations from other sources, including rat liver, has disclosed a group of 5-, 6-, and 7-member cyclic imides that are also substrates for enzymatic hydrolysis (9–11).

In accord with our interest in the imidase reaction, we sought to obtain an imidase from a mammalian source and to use it for an investigation of the mechanism of enzyme-catalyzed hydrolysis of imides. Spectrophotometric assays were devised to simplify exploration of the substrate spectrum; one assay, the hydrolysis of phthalimide (Reaction 2), was adopted for routine determination of enzyme activity. Our description is of a very broadly specific imidase purified to homogeneity from rat liver that displays stereoselectivity or stereospecificity for some substrates.

MATERIALS AND METHODS

With the exception of 6-sulfonylmethylidihydrouracil, a gift from O. Griffith (Medical College of Wisconsin), and of glutarimide and adipimide, gifts from J. H. Maguire (School of Pharmacy, University of North Carolina), reagents were purchased at the highest purity available. Candidate substrates for the enzyme were obtained from either Aldrich or Sigma; some of these compounds (2-phenylsuccinimide, 5-methylhydantoin, 5-isopropylhydantoin, adipimide, N-methylphthalimide, 2,3-pyridine dicarboximide) were from the Alfred Bader Library of rare chemicals (Aldrich). Antipain and pepstatin A were obtained from Peninsula Laboratories, and phenylmethylsulfonyl fluoride was from Pierce Chemical Co. Hydrazinopipericose-agarose (Spectra Gel/HA) was from Spectrum (Los Angeles).

Enzyme Assays

Standard Assay—A rapid spectrophotometric assay was devised in which the decrease in absorbance at 298 nm was measured upon hydrolysis of phthalimide as substrate (Reaction 2). A 2 mM solution of the sparingly soluble phthalimide was prepared in water and remained stable at 25 °C for several days. To start the reaction, 0.5 ml of this solution was added to 0.5 ml of 0.2 M MES* at pH 7.0 that contained an appropriate amount of enzyme. The reaction was monitored with a Hitachi 3110 spectrophotometer in a chamber maintained at 25 °C. Under these conditions, a change in Aₘ₉ of 2.26 represents the hydrolysis of 1 μmol of substrate; initial rates of change were a function of enzyme concentration over the absorbance range of 0.01 to 0.25/min at 298 nm.

A unit of activity is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of phthalimide/min. Specific activity is in terms of units of activity/mg of enzyme. Protein was determined with bicinchoninic acid using crystalline bovine serum albumin (Pentex) as standard (12).

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* The abbreviations used are: MES, 4-morpholinethanesulfonic acid; Bisce, N,N-bis(2-hydroxyethyl)glycine; CHES, 2-(cyclohexylamino)ethanesulfonic acid.
Other Substrates—The hydrolysis of a number of other cyclic imides were assayed spectrophotometrically in an entirely similar manner except for the wavelength used; necessary modifications of the assay are presented as needed for specific compounds.

Stereoselectivity and Isomerization

Hydrolysis of several substrates with a chiral center were monitored with a Jasco J-500C spectropolarimeter and the help of P. McPhie of this institute. Stereoselectivity of hydrolysis was determined by following the CD spectrum of the remaining unreactive or less reactive enantiomer. Reaction conditions were generally identical with those used for the spectrophotometric assays, except for the increased volume, 5 ml of the reaction vessel, of 0.2 M phenylmethylsulfonyl fluoride in 2-propanol, 2 mM pepstatin A and 2 mg/ml antipain; enzyme solutions were freshly concentrated to 1 millisiemens/cm or less with water (equal to a 10 mM potassium phosphate solution) before adding to a column (2.2 × 25 cm) of DEAE-Sepharose that had been equilibrated with buffer A containing 1 mM EDTA. Enzyme was eluted with a linear salt gradient that consisted of 250 ml of 10 mM Tris, 1 mM EDTA at pH 8.0 and 250 ml of the same solution supplemented to 0.1 M NaCl (Fig. 1C). Active fractions (3 ml each) were pooled (90 ml) and concentrated to 1.4 ml by ultrafiltration with a YM-10 membrane. The enzyme solution was concentrated to 1.8 ml by ultrafiltration, dispensed into vials in portions of 0.1 ml, and frozen in liquid nitrogen before storage at −80 °C.

Summary of the results of purification from 255 g of rat liver are presented in Table I.

RESULTS

Homogeneity and Size of Imidase—The imidase from rat liver was purified about 700-fold with a 4% yield to produce a homogeneous protein by the criterion of SDS-gel electrophoresis (Table I). A tediousely large number of procedures were necessary to remove the minor contaminants that were visible on SDS-gels until resolved at the last step by gel filtration. Despite the single and narrow band of protein observed upon SDS-gel electrophoresis (Fig. 1D), nondenaturing gels of the protein presented as broad bands covering about 15% of the length of a lane (not shown). Isoelectric

| Step | Volume | Total activity | Total protein | Specific activity |
|------|--------|----------------|---------------|------------------|
| 1. Extract | 1070 | 1300 | 29200 | 0.04 |
| 2. DEAE-cellulose | 1225 | 1200 | 7300 | 0.25 |
| 3. Polyelectrolyte | 275 | 1120 | 1850 | 0.60 |
| 4. DEAE-Sepharose | 465 | 780 | 360 | 2.2 |
| 5. Polyelectrolyte | 45 | 660 | 150 | 4.4 |
| 6. Isoelectric focusing | 55 | 440 | *a* | *a* |
| 7. Isoelectric focusing | 29 | 230 | *a* | *a* |
| 8. Hydroxyapatite | 330 | 20 | 13 | 1.3 |
| 9. Ultrafiltration | 20 | 115 | 10 | 12 |
| 10. DEAE-Sepharose/ EDTA | 110 | 88 | 6 | 15 |
| 11. Sephacryl S-300 | 8 | 46 | 1.7 | 27 |

*Protein could not be determined because of the presence of interfering amphoteries.
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FIG. 1. Results of purification. A, after isoelectric focusing; B, hydroxyapatite chromatography; C, DEAE-Sephacryl chromatography; D, upon evaluation of the purified enzyme by SDS-gel electrophoresis (lane 1, protein standards with size noted in kilodaltons; lane 2, 1 μg of the enzyme; lane 3, 10 μg of the enzyme). The following symbols are used: O, protein concentration; ●, imidase activity; □, ionic strength or pH.  mS, millisiemens.

Gel filtration with Sephacryl 300 allowed an estimate of Mr, the apparent molecular mass of 230,000 based on the assumptions of similar shape and partial specific volume, as compared with globular protein standards. By comparison with protein standards, an Mr of 53,000 was estimated by SDS-gel electrophoresis; the value was the same whether or not the enzyme was heated in the presence of 10 mM 2-mercaptoethanol. Thus, the enzyme is a tetramer of subunits of similar size that are not covalently joined.

When incubated at room temperature for 30 min, the enzyme appears to be most stable at pH 9.0, but the entire range from pH 6.6 to 9.0 leads to less than 15% loss of activity during this period. No loss of activity was sustained by storage at pH 7.5 for 6 months at −80 °C after freezing in liquid nitrogen.

Substrate Specificity—The enzyme catalyzes the hydrolysis of a wide variety of imides (Table II) that include five-, six-, and seven-member rings, as well as the open chain diacetamide. An amide (glycine 4-nitrophenyl anilid) and an ester (4-nitrophenyl acetate) were not substrates, nor were N-substituted imides such as N-hydroxyphthalimide or N-methyl phthalimide. A monoalkyl group on the carbon adjacent to the imide was acceptable, but dialkyl substitutions were not; 2-phenylsuccinimide was, and 2-phenyl-2-methylsuccinimide and 5,5-dimethylhydrazine were not substrates. Although an alkyl group on an adjacent nitrogen allowed hydrolysis, e.g. 1-methylhydrazine, negatively charged compounds such as dihydroorotic acid or 6-sulfonmethyl dihydrooracil were not substrates. The substituted ureas, 1-acetyl-3-methylurea and benzoyl urea, were not hydrolyzed, nor were 1-allantoin, barbituric acid, cis-1,2,3,6-tetrahydrophthalimide, and 2,4-thiazolidinedione.

The apparent Km for each of the substrates shown in Table III are in the millimolar range, similar to other enzymes active with the broad spectrum met in the detoxication process (14). Km values were high not only for the xenobiotics but also for the more physiological substrates dihydrouracil and dihydouracil (5-methylhydrazine). The products of these two physiological substrates were each identified as N-carbamyl-l-amino acids by the colorimetric method of Maguire and Dudley (15).

Reversibility—Imidase also catalyzed the experimental reversal of Reaction 1, i.e. dehydrolysis of N-carbamyl-α-alanine to dihydrouracil, as well as the analogous dehydrolysis of N-carbamyl-2,2-diaminopimelic acid to 5-methylhydantoin. Neither of the other available substrates for dehydrolysis, phthalamic acid and N-carbamylglycine, yielded the expected phthalimide and hydrazine, respectively. For N-carbamyl-α-alanine, dihydrouracil was the major product at pH 5.4 with an apparent Km of 2 mM and a Vmax of 0.5 mmol/min/mg of enzyme at that pH. The equilibrium constant for Reaction 1 was estimated to be 2.5 × 10-4/mmol from limited data, namely, the approach to equilibrium at pH 5.4 when large amounts of imidase were incubated with 5 mM and 10 mM N-carbamyl-α-alanine. At pH 5.4, the ratio of product to substrate at equilibrium was about 1 to 1.

Stereoactivity and Stereospecificity—Initial rates of hydrolysis of 6-methylhydrazine catalyzed by imidase at pH 7.0 or at pH 9.0 were similar when measured spectrophotometrically as a decrease in absorbance at 235 nm. As the reaction progressed at pH 9.0, the substrate was hydrolyzed, whereas the reaction appeared to approach only 50% of complete hydrolysis at pH 7.0 (Fig. 2A). An entirely similar result was observed for the hydrolysis of 5'-methylhydantoin (not shown). Both substrates have an optically active center as the result of the respective 6- and 5-methyl groups, and it is the preferential cleavage of one isomer at pH 7.0 that accounts for the differences in utilization. As presented in Fig. 2B for 6-methylhydrazine, the preferential use of one isomer is shown by the increase in circular dichroism at 230 nm as the reaction progressed. At pH 9.0, the optical activity reached a peak and then declined, i.e. both isomers were being utilized, but at different rates, which is an observation also supported by the approach to complete hydrolysis shown in Fig. 2A. Although absorbance appears to level off at 50% utilization at pH 7.0, prolonged incubation at pH 7.0 reveals an eventual decrease in circular dichroism consistent with the phenomenon of stereoactivity for both isomers rather than strict stereospecificity. Neither isomerization nor hydrolysis of 6-methylhydrazine were observed in the absence of enzyme.

Chiral compounds with larger constituents, examined here with 5-isopropylhydantoin and 2-phenylsuccinimide, were not hydrolyzed to completion. Only 50% hydrolysis was achieved with 5-isopropylhydantoin after prolonged incubation with imidase (Fig. 3), suggesting stereospecificity. Enzymatic hydrolysis of 2-phenylsuccinimide, however, is complicated by two factors, one of which is a small but significant spontaneous hydrolysis at alkaline pH. In addition, a base-catalyzed exchange in chirality occurs at position 2 of phenylsuccinimide, which was observed by NMR spectroscopy; deuterium from D2O exchanged readily with the hydrogen at position 2. The data shown in Fig. 4A for a solution of 10 mM 2-phenylsuccinimide in D2O and are compared with the same solution, containing 0.7 mM KOH (Fig. 4B) after incubation at room temperature. The decrease in the chemical shift at 4.3 ppm indicates the replacement by deuterium; additionally,
MgSO₄. The reaction was initiated by the addition of Phthalimide, 1, 298, 2.3, 33; 33, 298, 2.2, 14; 15, 2,3-Pyridine dicarboximide, 5, 290, 0.52, 21; 21, 290, 0.93; 9, 6-Methyl dihydrouracil, 5, 235, 0.4, 4; 4, 235, 0.42; 5, 4.7; 3,4-Pyridine dicarboximide, 4, 320, 0.46; 52, 52, 320, 0.93; 9, 5-Methyldihydrouracil, 5, 235, 0.37; 0.4; 0.35, 235, 0.37; 5-Methylhydantoin, 5, 230, 0.49; 7; 6, 242, 0.5; 7; 4.7, 2-Phenylsuccinimide, 5, 245, 0.35; 4.2; 3.8, 245, 0.49; 4, 4, 2-Methylhydantoin, 5, 230, 0.45; 12; 12, 230, 0.48; 18; 16, 5-Methylhydantoin, 5, 242, 0.49; 1.7; 1.6, 252, 0.52; 1.3; 1.2.

The exchange results in the loss of the coupling of protons and polarimetrically at 230 nm. pH 7.0; 2,3-pyridine dicarboximide, 50; (1.9 at pH 7.0); succinimide, 0.6; glutarimide, 1; adipimide, 5.

Glutarimide, 5, 220, 0.54; 0.2; 0.3, 230, 0.33; 2-Methylhydantoin, 5, 230, 0.49; 7; 6, 242, 0.5; 7; 7, 5-Methylhydantoin, 5, 242, 0.49; 1.7; 1.6, 252, 0.52; 1.3; 1.2.

The exchange results in the loss of the coupling of protons 3 and 3' from the proton of the neighboring carbon. Although carried out under basic conditions, a slow exchange of deuterium also occurs at neutrality.

For the imidase-catalyzed dehydration of N-carbamyl-DL-α-alanine, a mixture of enantiomers, the incubation solution shows an increase in residual optical activity at 235 nm (positive), due to the formation of 5-methylhydantoin from only one of the enantiomers. These findings are consistent with the observed appearance of a negatively rotating compound at that wavelength upon imidase-catalyzed hydrolysis of 5-methylhydantoin (Fig. 3B).

Substrate specificity

| Substrate                       | pH 7 | pH 9 |
|---------------------------------|------|------|
|                                 | λ    | µmol/min·mg | λ    | µmol/min·mg |
| Phthalimide                     | 1    | 298 2.3 | 1    | 298 2.2 |
| 3,4-Pyridine dicarboximide      | 4    | 320 0.46| 4    | 320 0.93|
| 2,3-Pyridine dicarboximide      | 5    | 290 0.52| 5    | 290 0.9  |
| Dihydrouracil                   | 5    | 235 0.34| 5    | 235 0.35|
| 6-Methyl dihydrouracil          | 5    | 235 0.37| 5    | 235 0.37|
| 5-Methyldihydrouracil           | 5    | 235 0.37| 5    | 235 0.37|
| Succinimide                     | 5    | 220 0.23| 5    | 230 0.52|
| 2-Phenylsuccinimide             | 5    | 245 0.35| 5    | 245 0.35|
| Glutarimide                     | 5    | 220 0.54| 5    | 220 0.54|
| Adipimide                       | 5    | 230 0.45| 5    | 230 0.45|
| Hydantoin                       | 5    | 230 0.49| 5    | 230 0.49|
| 5-Methylhydantoin               | 5    | 242 0.49| 5    | 242 0.49|
| 1-Methylhydantoin               | 5    | 242 0.49| 5    | 242 0.49|

Although dihydrouracil, hydantoin, and their derivatives are stable at alkaline pH, correction is required for spontaneous hydrolysis at pH 9.0 for the following compounds, with the rates presented in units of µmol/min:\n
- Phthalimide, 6.5
- 3,4-Pyridine dicarboximide, 50
- Succinimide, 0.6
- Glutarimide, 1
- Adipimide, 5

Effect of pH—The optimal pH for the several substrates examined appears to correlate qualitatively with the pKₐ of the amide proton. The pKₐ of each amide shown in Fig. 3 was determined by spectrophotometric titration as a function of pH and may be compared with the pH optimum found with imidase. In each instance, the pH optimum is slightly lower than the pKₐ. Not shown is 6-methylhydouracil, which has a pH and pH optimum similar to dihydrouracil.

Effect of Metals—Only the two physiological substrates, dihydrouracil and dihydrothymine, were hydrolyzed at a greater rate when in the presence of 5 mM MgSO₄ (Table III). Neither the specific activity of any of the other substrates tested, nor their Kₘ, was altered by magnesium ions (Table III). The Kₘ for MgSO₄ in the hydrolysis of dihydrouracil was 0.2 mM.

In searching for a dependence on zinc, such as that of the bovine liver metalloenzyme (6, 7), incubation of the rat imidase with 0.3 mM o-phenanthroline or with 3 mM ZnCl₂ was found to be without effect when either dihydrouracil or phthalimide were substrates.

N-Carbamyl-β-alanine as Inhibitor—Alone among the products of imide hydrolysis that have been tested, N-carbamyl-β-alanine served as a significant inhibitor of the enzyme. With 1 mM phthalimide (or with 1 mM 5-methylhydantoin) as the substrate at pH 7.0, 50 µM N-carbamyl-β-alanine inhibited 65% and, at pH 6.0, 90%; there was no inhibition at pH 8.5. Despite the stimulation of hydrolysis of dihydrouracil by Mg²⁺, the presence of Mg²⁺ appeared to have no effect on product inhibition by N-carbamyl-β-alanine. Nevertheless, consideration was given to the possibility that the metal ion might act only when N-carbamyl-β-alanine was generated on the enzyme, i.e., that magnesium's role was in the release of N-carbamyl-β-alanine from the enzyme after catalysis. A comparison was designed by which imidase, incubated in 1 mM N-carbamyl-β-alanine with or without 5 mM Mg²⁺ and then diluted 200-fold, was compared with another preparation in which the inhibitor was generated directly from dihydrouracil, with and without Mg²⁺ prior to dilution. The enzyme from each diluted preparation was then tested for activity.

![Graph](image-url)
FIG. 3. The extent of utilization by imidase of 5-isopropylhydantoin (□) and of 5-methylhydantoin (○), as measured spectroscopically at 325 nm (A) and polarimetrically at 230 nm (B).

FIG. 4. Proton chemical shifts from trimethylsilane of 2-phenylsuccinimide in D₂O without (A), and with (B), 0.7 mM KOH.

FIG. 5. pH activity relationships and correlation with the pKₐ of four imidase substrates. Activity for dihydrouracil was determined in the presence (+) and absence (×) of 5 mM MgSO₄; open (without Mg²⁺) and closed (with Mg²⁺) symbols are used for the other substrates.

with phthalimide as substrate in the absence of additional metal; the intent of the dilution step was to decrease the inhibitor concentration to a level at which phthalimide hydrolysis would not be inhibited by reason of normal product inhibition. All samples produced the same linear rate of phthalimide hydrolysis, except for the reaction mixture in which N-carbamyl-β-alanine had been formed directly from dihydrouracil in the absence of Mg²⁺. In the last instance, the 10-min lag that is seen (Fig. 6) is consistent with the suggestion that magnesium participates in the release of N-carbamyl-β-alanine from the enzyme, a process that occurs for the inhibited enzyme during the observed lag period.

DISCUSSION

The enzyme purified from rat liver that had been identified as dihydropyrimidinase is, in fact, an imidase of sufficiently broad specificity to include both cyclic and acyclic imides, and different in mechanism or selectivity from amidases and esterases. Unlike the zinc metalloenzyme from bovine liver (6, 7, 16), only magnesium appears to have an effect on the imidase from rat, and only with dihydrouracil and dihydrothymine as substrates.

We suggest that one basis by which imidase-catalyzed hydrolysis of imides is different from that of amides and esters is the capacity for the loss of the imide proton and subsequent formation of a partial planar intermediate (Scheme 1). The pH activity profiles for different imide substrates appear to correlate with their respective pKₐ values, in agreement with the loss of the imide proton as a necessary step (Fig. 5). Consonant with this conclusion is the enzyme's inability to utilize N-substituted imides.

Dihydrouracil, used here as a model compound, shows significant nonplanarity in its crystal structure, even for the extended s system along C4–N3–C2–N1 (17). The observation is expected, considering the strain on six-member rings that
arises due to the two sp² carbons (C5 and C6) in resisting the planarity produced by C4–N3–C2–N1 conjugation. The removal of the imide proton, however, forces the C4–N3–C2–N1 toward greater planarity in accommodating the delocalized electrons (18, 19). Evidence for such resonance structures includes the increase in absorption at about 235 nm at pH values near the pKₐ, a feature that was used here to determine the pK of some of the imides.

Dihydrouracil, hydantoin, and their derivatives resist chemical hydrolysis in alkaline solution, whereas succinimide and glutarimide, the analogous heterocycles with a carbon rather than nitrogen at the 1-position, are more labile under these conditions. For hydrolysis by the imidase, however, the order of activity is reversed, with dihydrouracil undergoing hydrolysis far faster than glutarimide. Although stability in alkaline solutions is enhanced by the presence of a greater number of resonance structures when nitrogen is at the 1-position, that same nitrogen serves as an electron sink for the addition of the incoming nucleophile. Enzyme is indicated as B in Scheme 1. The proposed structure 2 is also consistent with the significantly shorter bond distances observed at N1–C2 of both dihydrouracil and dihydrothymine (17, 20), which indicates strong conjugation between N1–C2–O2. Removal of the imide nitrogen from structure 1 also favors shortening bond distances of N3–C4 as the result of stronger overlap (19). Subsequent addition of water to C4 is driven by the two forces noted, i.e. attraction of water by positively charged species and the relief of ring strain. Hydrolysis is completed by N3-imide nitrogen from structure 1). The proposed structure 2 is also consistent with the

Acknowledgments—It is with pleasure that we acknowledge the valuable comments on the manuscript by William K. Berlin of this institute and by Owen W. Griffith of the Medical College of Wisconsin.

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